Expression and assembly of antibodies in transgenic plants

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ABSTRACT

Antibodies have important clinical applications in the diagnosis, management and treatment of diseases, as well as research uses. Many therapeutic antibodies are currently under development and a variety of systems have been investigated for their production. In 1989, transgenic plants were introduced as a new expression system for recombinant antibodies, with many potential advantages. In this thesis, some of the most important characteristics of plants have been examined, in order to understand the mechanisms and pathways of antibody production in plant cells. The findings have stimulated further investigations to determine the capacity of plants for expression of complex proteins.

Immunoglobulins are multimeric proteins with complex folding and assembly requirements. As newly translated polypeptides enter the endomembrane system of the cell, they interact with a series of molecular chaperones that control and facilitate these processes. The role of the plant HSP70 BiP homologue in this early process is demonstrated, mirroring the mechanism that takes place in mammalian cells. Within the endoplasmic reticulum and continuing in the Golgi apparatus, the assembled proteins undergo N-glycosylation, resulting in complex glycan structures attached to the amino acid backbone. This process was also demonstrated to occur in transgenic plants, with glycans attached to the same N-linked glycosylation sites used in mammalian cells.

In mammalian B and plasma cells, immunoglobulins are produced in secreted and membrane associated forms. In plants cells, IgG is normally secreted, but it was also demonstrated that the mammalian transmembrane sequence could be used to target and retain immunoglobulin heavy chain and fully assembled IgG in the plant cell membrane. Moreover, these findings were extended to a second and different type of membrane protein, CCR-5 a seven transmembrane chemokine receptor.

Overall, these findings serve to demonstrate the similarities between the plant and mammalian secretory pathways and provide a rationale for the efficient production of antibodies in plants. This was exemplified by the successful assembly and expression of a secretory antibody in plants. By co-expression of four transgenes, a decameric molecular complex was assembled in plants at levels of expression of up to 8 % of total soluble protein. These findings open the way to more widespread use of transgenic plants for expressing antibodies, particularly for applications such as passive immunisation for infectious diseases or cancer.

INTRODUCTION

It is more than a decade since the production of antibody was first described in transgenic plants (Hiatt *et al.*, 1989). However relatively little is still known about the plant intracellular mechanisms of protein folding and assembly that make this possible. Targeting expression of the recombinant molecules to the endomembrane system and secretory pathway of plant cells appears to be essential, and this is also the intracellular pathway for antibody synthesis in mammalian cells. Using recombinant antibodies as model proteins we have investigated the plant secretory pathway at a number of levels; by studying the interactions between immunoglobulin chains and endoplasmic reticulum (ER)-resident chaperones, determining whether immunoglobulins are correctly assembled, analysis of glycosylation, and by examining the default pathway of secretion. The findings allow us to explore further the potential of plants to express more complex proteins, namely membrane proteins and a monoclonal secretory IgA (SIgA) molecule.

SPECIFIC OBJECTIVES OF THE STUDY

1) Analysis of immunoglobulin assembly in plant cells and the role of ER-resident chaperones.

2) Purification of plant recombinant immunoglobulins for preliminary analysis of glycosylation.

3) Investigation of the targeting of mammalian proteins to the plant cell membrane, and to determine if functional proteins could be assembled and retained at this site.

4) Generation of a recombinant SIgA in plants.

5) Analysis of function of the plant SIgA and comparison with its' murine counterpart.

CHAPTER 1:

LITERATURE REVIEW

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CHAPTER 1: LITERATURE REVIEW

1. Monoclonal antibodies

The discovery of monoclonal antibodies (MAbs) (Kohler & Milstein, 1975) and their subsequent applications have revolutionised biological research. MAbs were initially used largely to study the size of the antibody repertoire and to examine the importance of somatic mutation in the generation of antibody diversity (Secher *et al.,* 1977). However because of some of their unique properties, such as specificity and purity, it soon became clear that they could be put to *in vitro* analytical and diagnostic purposes, and ultimately therapeutic uses.

Over 25 000 MAbs are currently commercially available, the majority of which have been generated in small amounts for laboratory-related research. Some MAbs have become commercially successful for the diagnosis and treatment of disease, and these may need to be produced on a larger scale. Currently, almost a quarter of all biotech drugs in development are MAbs, whilst licensed products are available to treat for example; alloimmune (e.g. OKT3) [Cosimi, 1983] and autoimmune (e.g. Infliximab) [Lipsky *et al.,* 1999] conditions, and for anti-tumour (e.g. Herceptin) [Baselga *et al.,* 1996], anti-platelet (e.g. Abciximab) [Hamm *et al.,* 1999], or anti-viral (e.g. Palivizumab) [Piedimonte *et al.,* 2000] activity (reviewed by Breedveld, 2000).

Advances in antibody engineering have also increased the potential number of applications for recombinant antibodies. Novel antibody-related proteins with desirable characteristics, such as molecules with reduced size or antibodies fused to enzymes, biological response modifiers or toxins can now be generated (Bookman, 1998; Shin *et al.,* 1993). In addition, the combination of phage display techniques and protein engineering technology has made it possible to isolate and express recombinant antibodies specific for almost any target antigen (McCafferty *et al.,* 1990; Winter *et al.,* 1994).

Although mammalian cells and bacterial expression systems are the most established methods for production, yeast and recombinant baculovirus-infected insect cell systems have also been used, although at present their applications appear to be more limited. Another means of generating recombinant proteins such as antibodies is by the use of transgenic animals, but this approach has disadvantages, and is sometimes restricted by legal and ethical opinion.

In the last fifteen years several groups have demonstrated that transgenic plants are an alternative system for expressing not only full-length antibodies (Hiatt *et al.*, 1989; Ma *et al.*, 1994), but also small antibody fragments (Benvenuto *et al.*, 1991) at relatively high levels. Further refinements in plant biotechnology may make it possible to grow novel immunotherapeutic reagents on an agricultural scale, reducing production costs significantly at the same time.

1.1 ANTIBODY STRUCTURE

Structure of IgG and IgA

A fully assembled IgG molecule comprises two heavy (H) and two light (L) polypeptide chains (HHLL), whose structure is maintained by non-covalent forces and by covalent intra- and inter-chain disulphide bonds between cysteine residues (Figure 1). The heavy and light chains are composed of variable and constant domains, which are structurally distinct and vary in their primary sequence. These domains are formed by the intra-chain disulphide links and consist of approximately 110 amino acids. The antigen-binding site is formed by the variable regions of the light and heavy chain, where it comprises a groove or cleft. The variable domains of both chains contain areas of hyper-variability that constitute the complementarity-determining regions (CDRs) and these form the predominant interactions with antigens (Jirholt *et al.*, 2001; Mian *et al.*, 1991). The domains in the carboxyl-terminus are relatively constant, and form the constant region (Fc), C_L on the light chain and C_H1, C_H2 and C_H3 on the heavy chain. In some antibody classes, for example IgM and IgG, this region of the heavy chain mediates the effector functions of phagocyte opsonisation and complement activation.

The heavy chains in the effector region are dimerised by two disulphide bonds, which create a hinge region. As shown in Figure 1, at the hinge region, the light chains are joined to the heavy chains by another disulphide bond. There are also many intramolecular disulphide bonds between amino acid residues within the light and heavy chains, as well as these described intermolecular disulphide bonds (reviewed by Rudd *et al.*, 1991).

The C_H1 and C_H2 regions are joined by a flexible hinge region, whilst the C_H2 and C_H3 domains consist of hydrophobic patches that form an accessible surface, or mini-hinge flexible region. Both these hinge regions are susceptible to attack by proteases, such as pepsin and papain (Mariani *et al.*, 1991; Kim *et al.*, 1995).

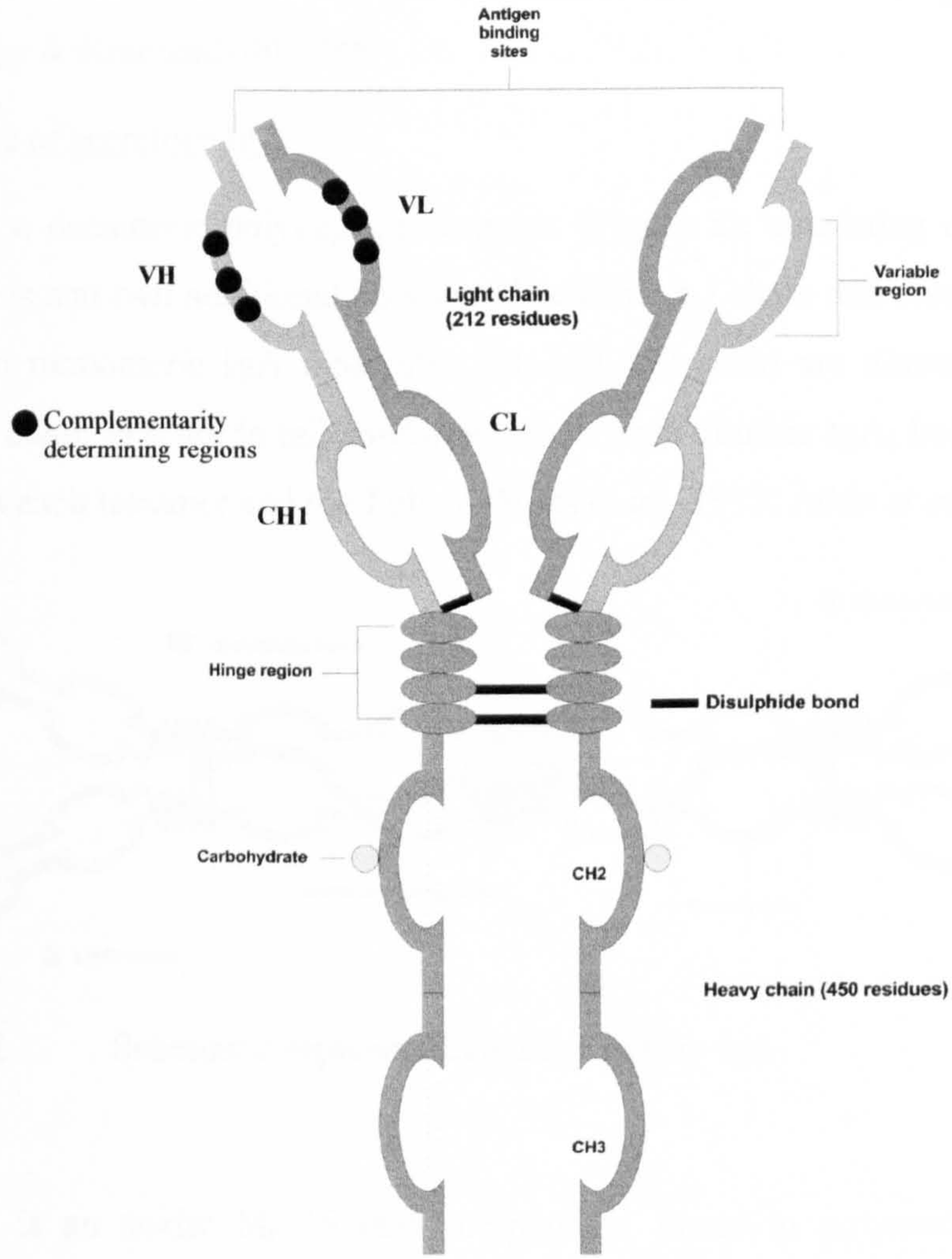

Figure 1. Schematic representation of an IgG molecule.

Each class of immunoglobulin has a characteristic type of heavy chain (α, δ, γ, ε and μ), and variations in the heavy chain amino acid sequence give rise to subclasses. Mammalian immunoglobulin α heavy chain consists of three constant-region domains (Cα1, Cα2, Cα3) [Hunkapiller & Hood, 1989]. In humans and rabbits, multiple α-chain isotypes have been identified (Spieker-Polet *et al.,* 1993), whereas in all other species only one IgA isotype is found. Two subclasses of IgA have been described in humans, IgA1 and IgA2. The major structural difference between these two subclasses lie in the hinge region, where the IgA2 molecule lacks a 16-amino acid sequence (Kawamura *et al.,* 1992), and in their carbohydrate composition (Wold *et al.,* 1995). The 16 amino acid sequence in IgA1 makes this molecule susceptible to bacterial IgA proteases, whereas IgA2 is resistant (Kilian *et al.,* 1996). The Cα3 domain on both sub-classes displays an 18-amino acid

tail-piece at its C-terminus which associates with J chain during the synthesis of IgA (Corthesy & Kraehenbuhl, 1999).

Structure of secretory IgA

SIgA is a decameric polypeptide complex (Figure 2), consisting of two monomeric IgA molecules and two additional polypeptide chains, a J chain and secretory component (SC). The two monomeric IgA molecules (M_r 160 000 each) are dimerised by a glycoprotein called J chain in a tail to tail conformation to form dimeric IgA, held by a disulphide bond between each tetramer and the J chain (Niles *et al.,* 1995; Atkin *et al.,* 1996).

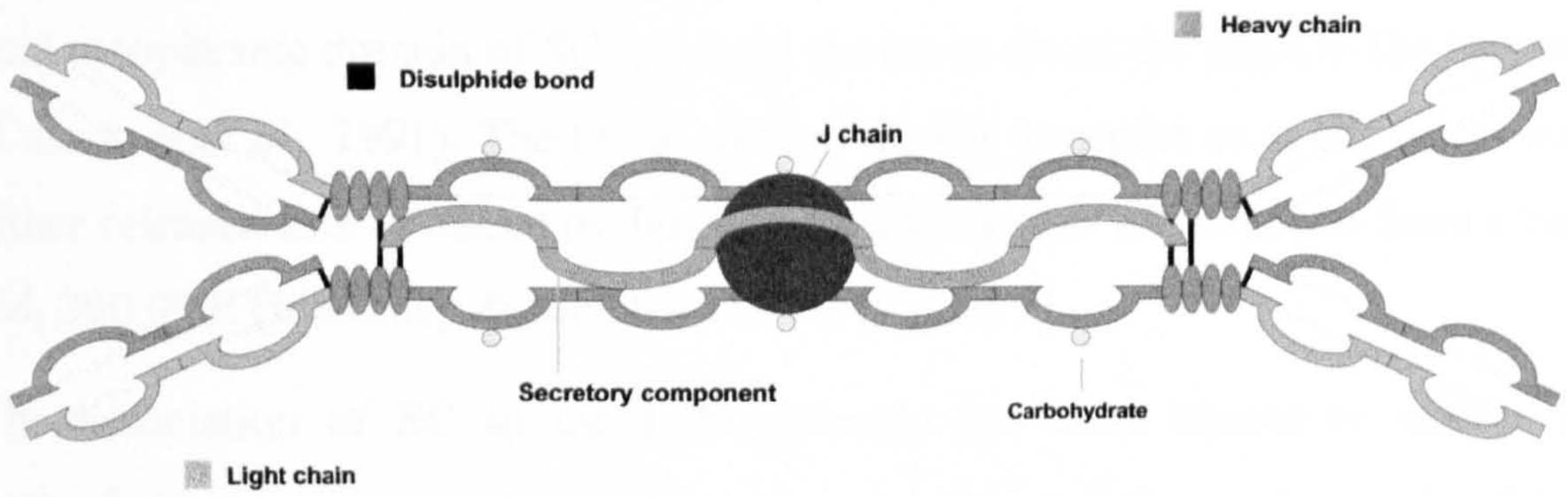

Figure 2. Schematic representation of secretory IgA.

J chain is an acidic M_r 15 000 polypeptide, found in polymeric immunoglobulins in covalent association with dimeric IgA and pentameric IgM molecules (Koshland, 1985). J chain is disulphide-bonded to two monomeric α heavy chains in human IgA, and can cause structural constraints in the Fc regions of polymeric immunoglobulins, determining a partial SC-binding site (Garcia-Pardo *et al.,* 1981).

The poly immunoglobulin receptor (pIgR) is comprised of five extracellular domains, comprising 110-120 amino acid residues that share homology with immunoglobulin domain sequences, a 23 amino acid membrane-spanning portion, and a cytoplasmic tail of about 100 amino acids (Mostov *et al.,* 1984). SC (M_r 80 000) represents the extracellular portion of the pIgR receptor. Human, rat, mouse, rabbit and bovine SC share extensive homology, especially in the first domain and in the cytoplasmic tail (Corthesy & Kraehenbuhl, 1999).

Rabbits have two pIgR primary translation products, as they also express a spliced version of the pIgR that lacks domains 2 and 3, but is fully competent for transport (Deitcher &

Mostov, 1986) and generates SC non-covalently bound to IgA (Frutiger *et al.*, 1987). This indicates that the information required for trafficking of the pIgR in epithelial cells resides within its cytoplasmic tail. In human SIgA, amino acids 14 - 38 in the N-terminus domain 1 of SC constitute the IgA binding epitope (Bakos *et al.*, 1991), and a cysteine residue at amino acid position 467 in the distal fifth domain is involved in disulphide bridge formation with Cys_{311} of one Cα2 domain of dimeric IgA.

The initial binding of SC to dimeric IgA *in vitro* is non-covalent, and subsequently inter-chain disulphide bridges are formed (Garcia-Pardo *et al.*, 1981). The three-dimensional structure of SC has not yet been fully determined. A 14 residue segment of the 103 amino acid cytoplasmic domain of SC has been shown to direct the pIgR to the basolateral surface (Casanova *et al.*, 1991). The SC is cleaved during transport or at the apical surface, and is either released free or bound to dimeric IgA (Mostov *et al.*, 1984) to form a SIgA antibody (M_r 390 000) (Mestecky & McGhee, 1987) [Figure 3].

The association of SC to the IgA molecule has been shown to confer resistance to proteolytic enzymes such as; Pronase, papain, trypsin, and pepsin (Underdown & Dorrington, 1974; Lindh, 1975). SIgA in duodenal secretions was shown to be more resistant than monomeric IgA to the action of proteases (Brown *et al.*, 1970). Furthermore, carbohydrate residues present in SC have been shown to anchor SIgA to mucus lining the epithelial surface (Phalipon *et al.*, 2002), an important role in SIgA-mediated immune exclusion *in vivo*.

1.2 GLYCOSYLATION OF IMMUNOGLOBULINS

A number of quality control mechanisms ensure that newly synthesised proteins attain their folded functional form. One important strategy is the covalent attachment of oligosaccharides to asparagine residues of nascent polypeptides, a process known as N-linked glycosylation. Thus, in the endoplasmic reticulum (ER) and the early secretory pathway, glycans play a crucial role in protein folding, oligomerisation, quality control, sorting and transport (Helenius & Aebi, 2001). The addition of oligosaccharides is beneficial during maturation as it makes folding intermediates more soluble (Rudd & Dwek, 1998). In addition, it permits some newly synthesised polypeptides to bind to the intracellular chaperones calreticulin and calnexin, which also play an important role in protein folding and quality control (reviewed by Helenius *et al.*, 1997).

The N-linked glycosylation of recombinant antibodies is an important step towards the production of fully functional immunoglobulins in any expression system. Whilst the N-linked glycosylation mechanism is conserved in mammalian and plant systems, there are differences in the fine details of oligosaccharide trimming and further modification of glycans in the Golgi apparatus (Driouich *et al.*, 1993).

The N-glycosylation process

N-linked oligosaccharides arise when blocks of 14 sugars are added to newly synthesised polypeptides in the ER. The N-linked glycans are attached to the side chain of asparagine residues in the consensus sequence Asn-X-Ser/Thr. The initial carbohydrate structure that is attached to nascent polypeptides contains two N-acetyl-glucosamine residues, nine mannose residues and three glucose residues (Figure 4).

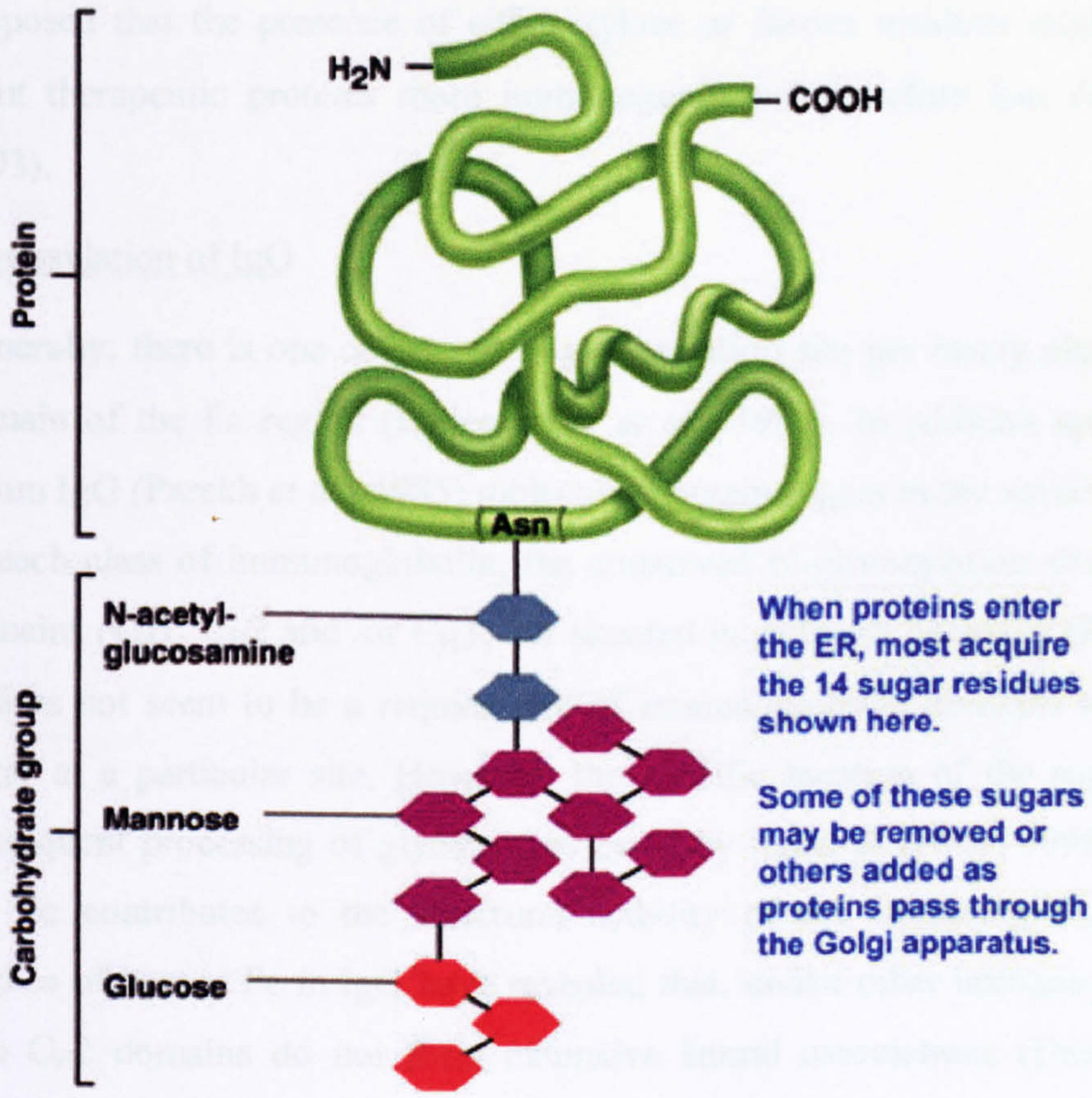

Figure 4. Initial N-linked oligosaccharide structures added to polypeptides in the ER.

Processing of N-linked glycans occurs along the secretory pathway as the glycoprotein travels from the ER to the Golgi apparatus towards its final destination. (Kornfeld & Kornfeld, 1985). Glycosidases and glucosyltransferases in the Golgi apparatus successively modify the oligosaccharide precursor to high-mannose type N-glycans, and then into complex type N-glycans.

Glycosylation in plants differs from that found in mammalian cells, although the high-mannose type N-glycans in plants have structures identical to those present in other eukaryotic cells. However plant complex N-linked glycans differ substantially (Driouich *et al.,* 1993). For instance, plants do not incorporate sialic acid, and their complex glycans are characterised by a β1,2-xylose residue linked to the β-mannose and an α1,3-fucose residue linked to the proximal glucosamine (Lerouge *et al.,* 1998). Larger, complex-type plant N-glycans are rare, but have been identified as additional 1,4-fucose and 1,3-galactose residues (for structures see Published Papers, Lerouge *et al.,* 1998 – page 369). It has been proposed that the presence of either xylose or fucose residues might make recombinant plant therapeutic proteins more immunogenic and therefore less desirable (Faye *et al.,* 1993).

Glycosylation of IgG

Generally, there is one conserved N-glycosylation site per heavy chain of IgG in the C_H2 domain of the Fc region (Rademacher *et al.,* 1986). In addition approximately 30 % of serum IgG (Parekh *et al.*, 1985) molecules contain sugars in the variable regions of the Fab. In each class of immunoglobulin, the conserved N-glycosylation sites on the heavy chain domains (C_H1, C_H2 and /or C_H3) are situated in different positions (Rudd & Dwek, 1997). It does not seem to be a requirement of immunoglobulin domains to contain a particular sugar at a particular site. However, the specific location of the sugar can influence the subsequent processing of glycans (reviewed by Rudd & Dwek, 1998). N-glycosylation of the Fc contributes to the structural stability of the immunoglobulin. Crystallographic studies of human Fc in IgG have revealed that, unlike other immunoglobulin domains, the two C_H2 domains do not form extensive lateral associations (Deisenhofer, 1981). The resulting interstitial region accommodates the oligosaccharides, attached to Asn_{297} on each heavy chain, so that the carbohydrates create a bridge across the domains (Parekh *et al.,* 1985). In IgG Fc, the hinge disulphide bond at Cys_{229} may provide a rationale for the large interstitial space between the two C_H2 domains. Non-covalent interactions between

the glycans and the Fc are also believed to help maintain the relative geometry of the C_H2 domains (reviewed by Rudd *et al.*, 1991).

Glycosylation of secretory IgA

The glycosylation pattern of SIgA is both complex and highly heterogenous (Endo *et al.*, 1994). The relative conformation of the two C_H2 domains of the IgA1 molecule is maintained by two inter-chain disulphide bonds at Cys $_{238}$ and Cys $_{239}$ (Mattu *et al.*, 1998). As the interstitial space is non-existent, larger sugars are fully exposed on the outside of the IgA1 molecule. Subsequently, nearly 90 % of IgA1 N-linked sugars are sialylated compared with just 15 % on IgG, which are found mainly on the more accessible sugars in the Fab' region (Wormald *et al.*, 1997).

An analysis of both the N- and O-glycans on the individual peptides from SIgA has recently been presented (Royle et *al.*, 2003). The IgA1 hinge region contains five O-linked glycans, that are recognised by the lectin jacalin, and which contribute to flexibility between Fc and $F(ab')_2$ fragments (Roque-Barreira & Campos-Neto, 1985). The tail-pieces of IgA (αtp) and IgM (μtp) both contain an N-linked glycosylation site, the presence of which is required for dimer formation in IgA (Atkin *et al.*, 1996). The N-linked carbohydrates present on both J chains and the α and μ tail-pieces play a role in polymerisation (Yoo *et al.*, 1999). The carbohydrate in μtp has been found to be required for the incorporation of J chain (Wiersma *et al.*, 1997). The N-glycan on the αtp of IgA1 is mostly a triantennary complex oligosaccharide (Mattu *et al.*, 1998), whereas IgM has a high mannose oligosaccharide carbohydrate in the tail-piece (Wormald *et al.*, 1991).

Human SC purified from milk is heavily glycosylated with between 5 and 7 N-linked sugar side chains that comprise more than 20 % of its molecular mass (Mizoguchi *et al.*, 1982).

Glycosylation and protein folding

Early investigations using tunicamycin, an inhibitor of glycosylation, showed that when N-glycan addition to proteins is blocked, most non-glycosylated forms of the proteins accumulate and are retained in the ER as aggregates (Olden *et al.*, 1982). This led to the concept that glycosylation assists protein folding by stabilising the conformation of the protein. In particular, the removal of glucose from the core N-linked glycans appears to indicate to the sorting apparatus in the luminal compartments, that the protein component is correctly folded (Hammond *et al.*, 1994; Fiedler & Simmons, 1995). These sorting steps are

now known to be mediated by the intracellular lectin-like chaperones calreticulin and calnexin, which specifically associate with monoglucosylated core glycans (reviewed by Helenius *et al.*, 1997).

The lectin interactions of calreticulin and calnexin detain monoglucosylated proteins in the ER until they are correctly folded; this is made possible by a cycle of glucosidase II action and reglucosylation by a lumenal UDP-glucose:glycoprotein glucosyltransferase, which produces the monoglucosylated form (Hebert *et al.*, 1995). This is an elegant system, as the glucosyltransferase acts preferentially on unfolded proteins (Sousa *et al.*, 1992), and once the folding process is over, the deglucosylated protein can leave the ER.

Function of Ig glycans

Glycosylation in antibody constant regions is important in stabilising antibody conformation and structure, in modifying the activity of effector functions, shielding the protein surface from proteases, and may also provide specific epitopes for recognition events (reviewed by Rudd & Dwek, 1998). This is despite the fact that the carbohydrate content of each isotype varies significantly (Torano *et al.*, 1977). Furthermore, phagocytosis, antigen-dependent cellular cytotoxicity, and the clearance and placental transfer of MAbs may be influenced by their glycosylation (Jefferis *et al.*, 1998).

Non-glycosylated IgG, expressed in tunicamycin-treated cells, was shown to be more susceptible to cleavage by papain than normal IgG, indicating that the conserved glycans in the Fc provide protection against proteolysis (Leatherbarrow & Dwek, 1984).

The presence of carbohydrate in the C_H2 domain of IgG is also required for Fc-mediated functions, such as complement activation and the engagement through Fc receptor binding of phagocytic cells (Nose & Wigzell, 1983; Tao & Morrison, 1989). Both non-glycosylated and degalactosylated IgG bind less efficiently to the Fcγ receptor (Nose & Wigzell, 1983). Aglycosyl IgG also demonstrates a loss of binding capacity to monocyte Fc receptors (Wright & Morrison, 1997).

In human IgA1, the effects of site-directed mutagenesis of the conserved N-linked glycosylation sites in the C_H2 region and secreted tail-piece has been studied in CHO (Chinese hamster ovary) cell lines to investigate their role in the structure and function of human IgA1 (Chuang & Morrison, 1997). The mutants showed a reduced affinity for their target antigen compared to wild-type IgA. Deletion of the carbohydrate had no effect on

the secretion of the antibody, with production of both monomeric and polymeric forms. This result differs significantly from that seen with murine IgA, in which the elimination of one or both of the N-linked glycosylation sites in the murine IgA C_H2 region resulted in a 95 % inhibition of secretion, as well as intracellular degradation of IgA protein (Taylor & Wall, 1988). The lack of the tail-piece carbohydrate increased the percentage of IgA present as polymers larger than dimers (Chuang & Morrison, 1997). In an earlier study, a lack of dimer formation was seen for IgA1 lacking the tail-piece carbohydrate, although this may have been accounted for by the low endogenous J chain production (Atkin *et al.*, 1996).

The effect of glycosylation on antigen binding has also been investigated (Donadel *et al.*, 1994). Cell lines making human monoclonal IgG, IgA and IgM were treated with tunicamycin to block N-linked glycosylation of the proteins. The native and non-glycosylated antibodies were then examined for their ability to bind different antigens. However in this case the absence of glycosylation did not result in the loss of antigen binding activity (Donadel *et al.*, 1994).

2. Immunoglobulin assembly

Immunoglobulins are normally synthesised by B cells and plasma cells. The immunoglobulin heavy (H) and light (L) chains are synthesised separately with N-terminal signal sequences. The signal sequences co-translationally direct the translocation of the nascent proteins to the lumen of the ER, where the signal sequences are cleaved by a signal peptidase (Von Heijne, 1984). Folding of both H and L chains occurs independently, and begins co-translationally (Bergman & Kuehl, 1979). The order of assembly for IgG molecules varies according to the isotype; for murine IgG1 the order is H, HH, HHL, then HHLL, although for IgG2b and IgM the order is H, HL, HHLL (Percy *et al.*, 1976). Disulphide bonds are formed with the assistance of the foldase enzyme protein disulphide isomerase (PDI) (Noiva & Lennarz, 1992), and a number of chaperones, such as the binding protein (BiP), calreticulin and glucose regulated protein 94 (GRP94) contribute towards the folding and assembly of immunoglobulins in the ER.

The assembly of dimeric IgA is initiated with the formation of H_2L_2 monomer units through a pathway in which $H + L \rightarrow HL \rightarrow H_2L_2$ (Chintalacharuvu *et al.*, 1994).

A conserved penultimate cysteine residue of the IgA tail-piece is involved in polymerisation (Wiersma *et al.*, 1997). The cysteine residue in the tail-piece of one chain forms a disulphide bond with J chain, which in turn forms a disulphide bond with the chain of a second monomeric sub-unit (Atkin *et al.*, 1996). However the tail-pieces alone do not determine the extent of polymerisation, as structural motifs in the constant region domains are also critical for polymer assembly and J chain incorporation (Yoo *et al.*, 1999; Sorensen *et al.*, 2000).

Dimerisation of IgA monomers by J chain occurs in mucosal plasma cells. Although all antibody-secreting cells produce J chain, it is incorporated only into polymeric immunoglobulins. This polymerisation not only increases the avidity of immunoglobulins for antigen, but it can also enhance effector functions such as complement activation and binding to Fc receptors in IgM (Smith & Morrison, 1994). In mammals, the J chain is added in plasma cells just prior to secretion (Parkhouse & Della Corte, 1973), and is essential for the production of SIgA, as J chain-deficient IgA present in secretions does not associate with SC (Brandtzaeg & Prydz, 1984).

The synthesis of SIgA requires a plasma cell secreting dimeric IgA (dIgA) as well as epithelial cells expressing the polymeric Ig receptor (pIgR). Normally, dIgA is transported across the epithelial barrier by the pIgR which is expressed at the basolateral surface of a variety of epithelial cells (Brandtzaeg, 1994). Following binding to the receptor, the receptor-ligand complex is internalised and transcytosed (Schaerer *et al.*, 1991), resulting in the release of the SIgA complex at the apical surface into the secretion (Mostov, 1994) – see Figure 5.

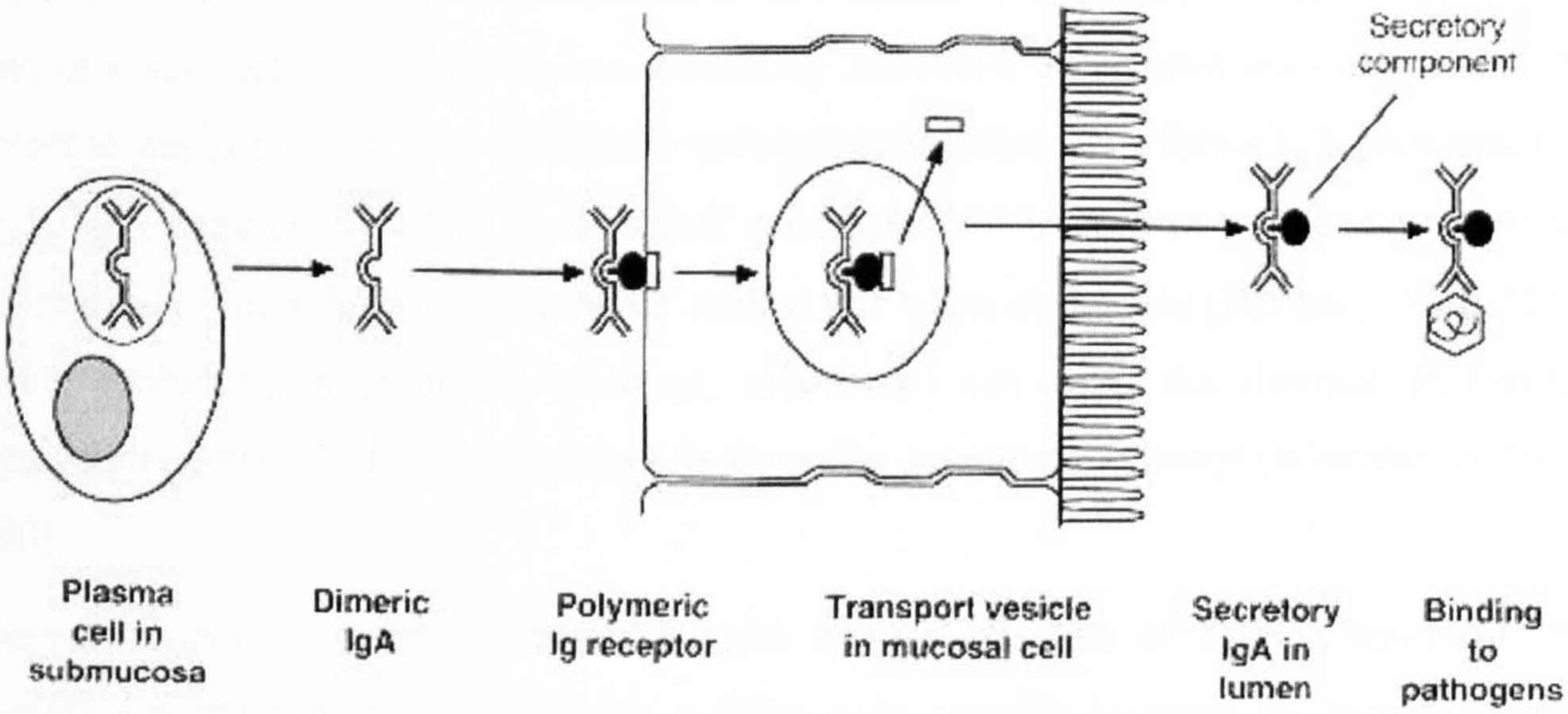

Figure 5. Schematic of secretory IgA assembly in mammalian cells.

The role of chaperones in the endoplasmic reticulum

The ER is responsible for the folding and quality control of secretory and membrane proteins. Protein folding has been described as "the process by which the linear information contained in the amino-acid sequence of a polypeptide gives rise to the well-defined three-dimensional conformation of the functional protein" (Hartl, 1996). Although newly synthesised proteins enter the ER in an unfolded conformation they depart fully folded (Gething & Sambrook, 1992). This process is not spontaneous, but is dependent on a set of conserved ER-resident proteins known as molecular chaperones, foldases or reticuloplasmins (reviewed by Hendrick & Hartl, 1993).

The ER differs from other cellular compartments, such as the cytosol or mitochondria, in several important respects. The lumen of the ER is an oxidising environment and is buffered by glutathione - which facilitates the formation of disulphide bonds (Hwang *et al.*, 1992) - and the ER lumen is the main cellular Ca^{2+} reservoir (Meldolesi & Pozzan, 1998). However whilst the complex and dense protein concentration in the ER lumen, estimated at more than 100 mg/ml, tends to promote aggregation and malfolding (Hartl, 1996), these are

the surroundings in which a battery of ER-resident chaperones control protein folding and perform the role of quality control.

One of the most important characteristics of ER-resident chaperones is their ability to bind to nascent substrates. Chaperones can transiently stabilise the unfolded state until either the correct folding or transport to a different cellular compartment is achieved (Zimmermann *et al.*, 1988; Cheng *et al.*, 1989; Goloubinoff *et al.*, 1989). Chaperones may also prevent the aggregation of malfolded proteins under normal and stress conditions (Pelham, 1986). If the correct conformation cannot be obtained, chaperones can target the aberrant protein for degradation, thus efficiently removing it from the secretory pathway (Klausner & Sitia, 1990).

Whereas chaperones may increase the yield but not the rate of folding reactions, the foldases are enzymes whose catalytic activity may actually increase the rate of protein folding (Gilbert, 1994). The most important chaperones and foldases with respect to immunoglobulin folding and assembly that have been identified in mammals are; the binding protein (BiP) [Haas & Wabl, 1983], glucose regulated protein (GRP94) [Melnick *et al.*, 1992], calreticulin (Sonnichsen *et al.*, 1994), calnexin (Bergeron *et al.*, 1994), and protein disulphide isomerase (PDI) [Freedman *et al.*, 1994]. Homologues for all the ER-resident chaperones have been identified in higher plants (reviewed by Boston *et al.*, 1996) and there appears to be a high degree of conservation between these molecules in plants, yeast and mammals (Mogelsvang & Simpson, 1998).

2.1 BINDING PROTEIN (BIP)

Immunoglobulin heavy chain sub-units, unlike light chains, are rarely exported as unassembled polypeptides following their synthesis in the ER. Intra-cytoplasmic heavy chains were found to be non-covalently associated with either light chains, or with a M_r 78 000 polypeptide in murine myeloma cells that did not synthesise immunoglobulin light chains (Haas & Wabl, 1983). This polypeptide was initially called immunoglobulin heavy chain binding protein (BiP), because of its ability to bind different heavy chain isotypes. BiP had also been independently identified as the glucose-regulating protein GRP78, which was induced by either a block in glucose synthesis or glucose deprivation in fibroblasts (Pouyssegur *et al.*, 1977), and was subsequently shown to be localised within the ER (Zala *et al.*, 1980).

A 736 base-pair BiP cDNA clone from a murine pre-B cell hybridoma was identified by the translation of hybrid-selected mRNA, which yielded a single polypeptide of expected size (Haas & Meo, 1988). The C-terminal 142 amino acids of this BiP clone were sequenced, revealing a tetrapeptide Lys-Asp-Glu-Leu (KDEL) characteristic of soluble proteins retained in the ER lumen. BiP-related RNA transcripts were shown to be constitutively expressed in various murine and rat tissues, although only a single copy of the BiP gene was detected in mouse and rat. This finding suggested that in addition to its' association with immunoglobulin heavy chains, BiP might have a more widespread function.

Subsequently BiP has been shown to bind transiently to a variety of nascent secretory and transmembrane proteins, and to remain permanently bound to malfolded proteins that accumulate within the ER (Hendershot *et al.*, 1987). The hypothesis that the presence of malfolded proteins may be the primary signal for the induction of BiP was tested by expressing wild-type and mutant forms of influenza virus haemagglutinin in simian cells (Kozutsumi *et al.*, 1988). Only malfolded haemagglutinins, whose export from the ER was blocked, induced BiP mRNA accompanied by an increase in synthesis of BiP protein.

BiP is the most abundant of the ER-resident chaperones (Gething & Sambrook, 1992), and belongs to the M_r 70 heat-shock (HSP70) family of proteins. HSP70s are highly conserved among different eukaryotes and have members in every cellular compartment in which protein folding takes place (Munro & Pelham, 1986). HSP70 genes can be classified into two groups: cognate genes (HSC70) which are expressed under normal growth conditions (and not strongly induced by heat shock), and those that are not expressed under normal conditions but are heat-shock inducible (Lindquist & Craig, 1988).

Studies with wild-type and mutant yeast strains deficient in the yeast *kar2* gene homologue of BiP have shown that BiP is required for the translocation and correct folding of newly synthesised proteins in the ER (Sanders *et al.*, 1992; McClellan *et al.*, 1998), as well as for retrograde transport across the membrane of aberrant proteins destined for degradation by the proteasome (Plemper *et al.*, 1997). Evidence for a similar role for BiP in plants has been obtained with the partial complementation of a temperature sensitive yeast *kar2* mutant by a tobacco BiP cDNA (Denecke *et al.*, 1991). This evidence suggests that amino acid residues important for BiP function in yeast may be evolutionarily conserved between yeast and higher plants.

Several studies have highlighted the transient association of BiP with mammalian immunoglobulin heavy and light chains during their folding and assembly (Bole *et al.*, 1986; Hendershot *et al.*, 1987; Knittler & Haas, 1992). BiP was shown to bind more permanently to misfolded, underglycosylated or unassembled proteins whose transport from the ER was blocked (Gething & Sambrook, 1992). BiP is also involved in maintaining the permeability barrier of the ER membrane, both before and early on in the translocation process (Hamman *et al.*, 1998; Gething, 1999).

Recognition of polypeptide substrates by BiP

The chaperone activity of BiP in the ER lumen is dependent on its ability to recognise and discriminate between a variety of properly folded and unfolded structures. The peptide-binding site of BiP can be filled by 7 amino acid residues, and preferentially binds aliphatic side chains, which are typically present inside folded proteins (Flynn *et al.*, 1991). A heptameric motif, Hy-(W/X)-Hy-X-Hy-X-Hy, has been identified where Hy is a bulky aromatic or hydrophobic residue, W is tryptophan, and X is any amino acid (Blond-Elguindi *et al.*, 1993b). Further, a computer-based scoring system was devised to help predict BiP-binding sites in natural proteins (Blond-Elguindi *et al.*, 1993b). This technique demonstrated that BiP-binding sites in immunoglobulins were likely to be concentrated in sequences that participate in contact sites between the heavy and light chains, and that hydrophobic residues destined to become buried in the interface are disproportionately present within the potential BiP-binding sites (Knarr *et al.*, 1995). In binding to hydrophobic surface regions on the isolated sub-units, BiP may prevent inappropriate interactions with other partially folded polypeptides. In this way, non-specific aggregation and unproductive side reactions would be suppressed (Knarr *et al.*, 1995).

BiP and immunoglobulin assembly

In the course of plasma cell development, immunoglobulin heavy chains are synthesised before light chain genes are rearranged and expressed (Taussig, 1988). Immunoglobulin heavy chains form homodimers in pre-B cells (Kaloff & Haas, 1995), but in the absence of light chains they are retained in the ER through an association with BiP (Haas & Wabl, 1983; Bole *et al.*, 1986). Initiation of light chain protein expression permits the assembly of complete immunoglobulin molecules, which takes place in the ER. However incompletely

or incorrectly assembled immunoglobulin molecules remain bound to BiP and are ultimately targeted for degradation (Gardner *et al.*, 1993).

Immunoglobulin light chains

Immunoglobulin light chains are usually secreted (Baumal *et al.*, 1973), however in some instances the export of light chains from the ER is reliant upon association with heavy chains (Kohler *et al.*, 1976; Oi *et al.*, 1983). It was subsequently reported that these light chains were bound to BiP (Dul & Argon, 1990). However these light chains do not belong to the 'malfolded' type of BiP ligand, as they are assembled into antibody molecules when heavy chains are coexpressed. It was suggested that the association of BiP with light chains might be a general event, in which stable complexes are formed in cells which do not secrete their light chains, and transient complexes formed in cells which do (Knittler & Haas, 1992).

BiP binds transiently to nascent immunoglobulin light chains *in vivo* when the variable region is unfolded, and is released as the domain folds (Hendershot *et al.*, 1996). BiP-binding to a murine lambda light chain during folding was investigated *in vivo* using BiP ATPase mutants which bind irreversibly (Hellman *et al.*, 1999). The lambda light chain is composed of two immunoglobulin domains that fold independently of one another, each containing multiple potential BiP-binding sites. Although both the wild-type and mutant BiP associated with the unoxidised variable region domain, neither BiP protein was found to bind to the constant region domain. Subsequent *in vitro* and *in vivo* folding analyses demonstrated that the constant domain folds rapidly and stably even in the absence of an intra-domain disulphide bond. These findings suggest that it is the rate and stability of protein folding that determines whether or not a particular BiP-binding site is recognised. The presence of a BiP-binding site on a nascent chain does not ensure that BiP will bind and become involved its' folding, and it appears that BiP preferentially binds to proteins that fold slowly or unstably (Hellman *et al.*, 1999).

Immunoglobulin heavy chains

Indirect evidence suggested that the first constant (C_H1) domain of the immunoglobulin heavy chain was required for association with BiP (Hendershot *et al.*, 1987). Whereas mutant heavy chains lacking either C_H2 or C_H3 domains remained in the ER complexed with BiP, chains which lacked C_H1 were transported along the secretory pathway and did

not appear to interact with BiP (Hendershot *et al.*, 1987). It was also suggested that sequences within the V_H domain may influence the association of immunoglobulin heavy chains with BiP (Haas, 1991).

By using a C_H1 domain-deleted heavy chain, BiP was shown to bind to other heavy chain domains, although these interactions were transient and restricted to unoxidised heavy chains (Kaloff & Haas, 1995). The first constant domain (C_H1) was thus revealed as the stable binding site on unassembled heavy chains. Whilst it is not obvious why BiP should bind stably to the C_H1 immunoglobulin domain and bind only transiently with other domains, two possible explanations have been proposed. The first is that BiP may bind to linear hydrophobic regions on nascent chains before they fold, such as V_L, in addition to hydrophobic faces on folded protein sub-units prior to their assembly into multimers, for example to C_H1 of heavy chain (Lee *et al.*, 1999). The second explanation is that BiP may bind to unfolded areas of the proteins if the C_H1 domain is unable to fold until it pairs successfully with a light chain partner (Lee *et al.*, 1999). Light chains are required *in vivo* to facilitate the folding of the C_H1 domain and the release of BiP (Lee *et al.*, 1999; Vanhove *et al.*, 2001).

<u>Regulation of BiP function</u>

Mammalian BiP exists in interconvertible monomeric and oligomeric forms *in vivo* that can be modified by phosphorylation (Hendershot *et al.*, 1988) and by ADP-ribosylation (Leno & Ledford, 1990). There is some evidence that these post-translational modifications may be important for the regulation of both the synthesis and polypeptide-binding activity of BiP. Conditions that increase the level of unfolded polypeptides in the ER lumen, such as heat shock and glucose starvation, may lead to reduced post-translational modification of BiP (Carlsson & Lazarides, 1983; Laitusis *et al.*, 1999) and an increase in the proportion of the monomeric form (Freiden *et al.*, 1992). As only the unmodified, monomeric BiP molecules are found in complexes with unassembled or unfolded proteins, it has been suggested that post-translational modifications of BiP may provide a means of tapping a reservoir of BiP when it is needed (Gething, 1999).

Binding affinity for ATP and a weak ATPase activity is a common property of all HSP70's (Gething & Sambrook, 1992). In turn, the affinity of the C-terminal domain of BiP for polypeptides is dependent on whether the N-terminal nucleotide-binding site is occupied by

ATP (low affinity but rapid binding) or ADP (high affinity but slow exchange). Binding of a polypeptide to the C-terminal domain increases the rate of ATP hydrolysis by the N-terminal ATPase domain (Flynn *et al.*, 1989; Blond-Elguindi *et al.*, 1993a).

In vitro, proteins associated with BiP can be released by the addition of ATP to the complex (Dorner *et al.*, 1990; D'Amico *et al.*, 1992). The use of ATP to reverse the binding of HSP70s to their substrates has been used as a diagnostic assay for chaperone activity (Nandan *et al.*, 1994).

<u>BiP in plants</u>

BiP is the best characterised of the ER-resident chaperones in plants, having been identified in a number of different plant species, including tobacco, maize, barley, rice, *Arabidopsis*, spinach, tomato and soybean (reviewed by Boston *et al.*, 1996). In all plant systems examined so far, BiP accumulates following treatment with tunicamycin, and during glucose starvation (D'Amico *et al.*, 1992). Tunicamycin is an inhibitor of N-linked glycosylation which causes the accumulation of misfolded proteins in the ER of both plant cells and mammalian cells (Kozutsumi *et al.*, 1988) and results in the increased transcription of several chaperones, including BiP and PDI.

Like yeast and mammalian BiP, spinach BiP has been reported to be encoded by a single gene (Anderson *et al.*, 1994). In contrast, several highly conserved isoforms of BiP which are encoded by multigene families have been identified in tobacco, maize and soybean (Denecke *et al.*, 1991; Fontes *et al.*, 1991). In tobacco, eight cDNA clones of the BiP homologue have been isolated, and six were found to be distinct from each other at the nucleotide sequence level (Denecke *et al.*, 1991). However most of these nucleotide substitutions did not alter the encoded amino acids. The signal sequence and in particular the C-terminal part that precedes the HDEL sequence were shown to be the most divergent portions of the protein sequence. The biological significance of differences in gene number with respect to gene regulation and possible redundancy of function has not yet been explained.

All plant BiPs identified so far contain the tetrapeptide C-terminal ER retention signal HDEL (Vitale *et al.*, 1993). There is no evidence to suggest that either lumenal BiP or its cytosolic homologue are heat-shock inducible in plants (Denecke *et al.*, 1991; D'Amico *et al.*, 1992; Walther-Larsen *et al.*, 1993). The amino acid sequence of one of the members of

tobacco BiP (BLP4) [Denecke *et al.*, 1991] has been compared with that of hamster BiP (Ting *et al.*, 1987) and yeast BiP (Normington *et al.*, 1989; Rose *et al.*, 1989). The alignment studies demonstrated that tobacco BiP has 76 % homology with mammalian BiP, and 63 % with yeast BiP.

Some of the initial investigations into BiP in plants focused on whether the BiP genes were expressed in a tissue-specific manner. RNA was extracted from different tissues at several developmental stages from tobacco plants (Denecke *et al.*, 1991). BiP mRNA was barely detectable in leaves, and was present at only slightly higher levels in roots. BiP mRNA levels were modest in stems and petals, but higher BiP mRNA levels were detected in flower organs that have secretory tissue, such as the anthers, stigma and style. Of all the tissues analysed, germinating seeds 4 days after imbition had the highest BiP mRNA level. BiP mRNA levels thus appeared to be most abundant in tissues with high proportions of rapidly dividing cells, containing large amounts of ER membranes (Denecke *et al.*, 1991). These observations support the theory that BiP is required in the folding process of *de novo* synthesised secretory proteins in unstressed cells.

The stress-related expression pattern of BiP was examined using germinating tobacco seeds shaken in culture medium at 24°C (Denecke *et al.*, 1991). Seeds were incubated in the presence of either 20 µg/ml tunicamycin or 5 µM calcium ionophore A23187 for 16 hours under standard conditions. In addition, seeds were also subjected to cold shock treatment at 4°C, and heat shock treatment at 42°C. In each case, total RNA was extracted from protoplasts prepared from seeds, and BiP mRNA levels were examined by Northern blotting. BiP mRNA levels were elevated in tobacco protoplasts treated with tunicamycin. However it is unclear whether the expression of BiP in plant cells is induced directly by the presence of misfolded proteins in the lumen of the ER, or by the abnormal glycosylation pattern of proteins. In contrast to mammals, tobacco BiP mRNA levels were not elevated by treatment with the calcium ionophore A23187. This observation reflects the fact that calcium ionophores do not affect the retention of chaperones in tobacco, and so obviates the need for *de novo* BiP synthesis to maintain homeostasis. In order to investigate the role of BiP in the transport and secretion of proteins in plants, transgenic tobacco was produced with altered BiP levels (Leborgne-Castel *et al.*, 1999). Two chimeric genes were generated; a wild-type BiP coding region under the transcriptional control of the CaMV 35S promoter, and an antisense construct in which the BiP coding region was in the reverse orientation.

The stable over-expression of BiP protein in tobacco plants was confirmed by Western blotting of plant extracts (Denecke *et al.*, 1991). Whereas BiP mRNA levels were increased as much as 150-fold, protein levels were only increased by 5-fold. The large amount of degradation products that correlated with over-expression of BiP suggests that a higher turnover of BiP occurs when BiP is too abundant. However the pathway for the disposal of excess BiP has not been clearly identified (Leborgne-Castel *et al.*, 1999).

No clear phenotypes were visible with the mature BiP antisense plants, although freshly cut plants took longer to produce roots than cuttings from non-transformed plants or BiP-overproducers (Leborgne-Castel *et al.*, 1999). In an additional experiment, tobacco BY2 suspension cultures (Gomord *et al.*, 1998) were transformed with the BiP antisense construct. Although it was not possible to generate plants containing significantly reduced BiP levels by this antisense inhibition strategy, the number of resistant calli was dramatically reduced, suggesting that even small reductions in BiP levels can have a detrimental effect on tobacco cell viability.

In order to examine the effect of artificially increasing BiP protein levels on the unfolded protein response (UPR) (Shamu *et al.*, 1994), protoplasts were prepared from non-transformed plants and BiP-overproducers (Leborgne-Castel *et al.*, 1999). One population was treated for 2.5 hours with tunicamycin (20 μ g/ml). The basal transcript levels of endogenous BiP genes were shown to be downregulated in BiP overexpressers. The level of BiP mRNA induced by tunicamycin in non-transformed cells was indistinguishable from that in transformed cells overproducing BiP which were subjected to the same treatment. Thus artificially increasing BiP protein levels results in a downregulation of the UPR, as observed in CHO cells (Dorner *et al.*, 1992) and in yeast (Kohno *et al.*, 1993). This suggests that BiP transcription may be regulated by a feedback mechanism that involves the monitoring of BiP protein levels. Hybridisation using a calreticulin-specific probe revealed a similar reduction in the basal calreticulin mRNA levels, and demonstrated that the regulatory effect of BiP extends to other ER chaperones (Leborgne-Castel *et al.*, 1999).

2.2 CALRETICULIN

Calreticulin was first identified as a Ca^{2+}-binding protein (Ostwald & MacLennan, 1974). More recent evidence confirmed that calreticulin is one of the major Ca^{2+}-binding proteins in the ER (Michalak *et al.*, 1992), which plays an important role in the regulation of intracellular Ca^{2+} ion homeostasis by modulating Ca^{2+} storage and transport (Berridge, 1993; Meldolesi & Pozzan, 1998). Furthermore, calreticulin also acts as a highly versatile lectin-like ER chaperone, that participates during the synthesis of a variety of molecules such as MHC Class I and HIV gp120 (Michalak *et al.*, 1999, Danilczyk *et al.*, 2000, Otteken & Moss, 1996).

Calreticulin (M_r 46 000) is a soluble analogue of calnexin that resides within the ER lumen (Nauseef *et al.*, 1995; Otteken & Moss, 1996). Both calreticulin and calnexin contain a lectin-binding site that directs their association with newly synthesised glycoproteins. Although many glycoproteins bind to both calreticulin and calnexin, there are differences in the range of glycoproteins that each binds. Both calreticulin and calnexin have homologues in a diverse range of plant and animal species (Boston *et al.*, 1996; Krause & Michalak, 1997; Michalak *et al.*, 1999).

Calreticulin, calnexin and glycoprotein folding

The working principle of many chaperones, such as BiP, depends upon conformational changes driven by the hydrolysis of ATP. However the binding and release of calreticulin and calnexin relies upon their interaction with newly synthesised glycoproteins that have undergone partial enzymatic trimming of their core N-linked glycoproteins (Hammond & Helenius, 1995). Release from calreticulin and calnexin takes place when the polypeptides have undergone oligomeric assembly or attained a fully folded conformation (Hammond & Helenius, 1995). In this way both chaperones play a crucial role in the quality control of glycoprotein folding.

The association of calreticulin and calnexin with glycoproteins may be a transient step that begins during early maturation and continues post-translationally for variable periods of time, ranging from a few minutes to several hours. However with unassembled sub-units of oligomers, or permanently misfolded proteins, chaperone association may persist until the protein is degraded (reviewed by Helenius *et al.*, 1997).

Indirect evidence suggests that the binding of calreticulin and calnexin also involves protein-protein interactions. For example, some glycoproteins deprived of glycan addition have been shown to co-immmmunoprecipitate with calnexin (Loo & Clarke, 1994). It was also observed that class-I heavy chains may be chemically cross-linked to calnexin at a site close to the transmembrane region (Margolese *et al.*, 1993). It is possible that oligosaccharides serve to bring the substrate and calnexin into close proximity and thereafter a protein-protein interaction, typical of classical chaperones, takes place (reviewed by Helenius *et al.*, 1997).

As the lectin specificities of calreticulin and calnexin are identical, there is extensive overlap between their substrate glycoproteins (Ware *et al.*, 1995; Spiro *et al.*, 1996), and in some cases they can even associate simultaneously with the same glycoprotein (Otteken & Moss, 1996). However it has been shown that the vesicular stomatitis virus (VSV) G glycoprotein binds to calnexin but not to calreticulin (Peterson *et al.*, 1995). It was also demonstrated that calreticulin dissociates more rapidly than calnexin as the folding of the T cell receptor (Van Leeuwen & Kearse, 1996), and influenza virus haemagglutinin proceeds (Hebert *et al.*, 1997). Mutation of glycosylation consensus sites of influenza haemagglutinin demonstrated that the location of N-linked glycans plays a role in differential calnexin and calreticulin binding (Hebert *et al.*, 1996).

Calreticulin and calnexin in plants

Relatively little is known about the lectin-like activity of calreticulin and calnexin in plants. Some evidence that glycans are involved in the folding process came from the finding that assembly of phaseolin, a seed storage protein, occurs at a faster rate when glucose trimming by ER glucosidases is inhibited (Lupattelli *et al.*, 1997).

The first calnexin-like protein reported in plants was identified in *Arabidopsis thaliana* (Huang *et al.*, 1993). Subsequently, calnexin has been isolated from soybean and maize (Goode *et al.*, 1995). Although the role of calnexin has mainly been studied in human and mouse cells, the high degree of conservation identified suggests that it might function similarly in plants.

A calreticulin-like protein, of approximately 55 kDa, was initially purified from spinach leaves (Menegazzi *et al.*, 1993). Since then, calreticulin has been cloned from several other angiosperms, including barley, in which two isoforms were detected with Ca^{2+}-binding

activity similar to that reported for mammalian calreticulin (Chen *et al.*, 1994). Subsequently, calreticulin was shown to be a marker protein for the ER in tobacco (Denecke *et al.*, 1995). Calreticulin has also been identified in maize (Dresselhaus *et al.*, 1996) and soybean (Goode *et al.*, 1995), and is the major calcium storage protein in the ER of pea plants (Hassan *et al.*, 1995). More recently calreticulin was characterised in *Ginkgo biloba*, a plant considered to have one of the earliest gymnosperm lineages (Nardi *et al.*, 1998).

The involvement of calnexin in protein folding and oligomerisation in plants was raised by evidence that it associates with a vacuolar H^+-translocating ATPase, which is a membrane spanning protein (Li *et al.*, 1998). Calnexin and BiP were found in association with this ATPase from oat seedlings, and the presence of calreticulin was not excluded (Li *et al.*, 1998). It is unclear whether there is a BiP/calnexin/nascent ATPase complex, or whether BiP and calreticulin bind successively to the folding intermediates of the ATPase.

Relative levels of calreticulin expression have been investigated in different tobacco tissues, and were found to be similar to that of the ER-resident chaperone BiP (Denecke *et al.*, 1995). For both transcripts the lowest level was observed in leaves. Higher levels were detected in roots and stems, and the highest abundance was found in floral tissues and germinating seeds, both of which contain cells with high secretory activity. Experiments using tobacco cotyledon cells demonstrated that calreticulin was localised in the ER lumen of germinating tobacco seeds (Denecke *et al.*, 1995).

Using RNA extracted from tobacco protoplasts incubated in culture medium containing tunicamycin (20 μg/ml) for up to 9 hours, expression patterns for calreticulin dissimilar to those of other ER-resident proteins were reported (Denecke *et al.*, 1995). Transcripts of calreticulin, BiP, and PDI were detected, and whereas tunicamycin treatment mainly induced BiP and PDI, the effect on calreticulin expression was much less (Denecke *et al.*, 1995). Thus, whereas BiP and PDI may be regulated by the UPR feedback mechanism, it appears that alternative signal transduction pathways might exist for calreticulin. This could explain how other stress and hormone treatments resulted in the enhanced synthesis of different ER chaperones (Denecke *et al.*, 1995).

The tobacco homologue of calreticulin was identified in protein complexes isolated from tobacco cells *in vivo* (Denecke *et al.*, 1995). The most abundant complex formed by

calreticulin also involved a M_r 75 000 protein that was found to be associated even in the absence of ER stress (Denecke *et al.*, 1995). Subsequently this protein was identified as BiP, although the presence of this novel BiP-calreticulin complex cannot be explained by the established lectin-binding activity of calreticulin (Spiro *et al.*, 1996), as BiP is not a glycoprotein. Although the BiP-calreticulin complex was disrupted by ATP, calreticulin associated with BiP was neither unfolded nor partially or improperly folded (Crofts *et al.*, 1998). Pulse-chase experiments were used to demonstrate that the BiP-calreticulin complex was stable and not due to the presence of transiently unfolded calreticulin. Additional studies showed that the interaction appears to involve a very large proportion of the cellular BiP compared with a small proportion of cellular calreticulin. Immunoprecipitations performed with extracts from *in vivo* labeled protoplasts isolated from BiP-overexpressing plants showed additional recruitment of BiP to the complex with calreticulin, as observed by a higher BiP/calreticulin ratio (Crofts *et al.*, 1998). In contrast, calreticulin over-expression led to an increase in non-complexed calreticulin, and demonstrated that it was the level of BiP that determined the degree of complex formation with calreticulin.

Binding of BiP to calreticulin was also compared with the binding of BiP to an assembly-defective protein. Transgenic plants were used which overproduce a mutant phaseolin monomer, a model ligand for BiP (Pedrazzini *et al.*, 1994;1997). Protoplasts were pulse labeled and immunoprecipitations performed with anti-phaseolin, anti-BiP, or anti-calreticulin antisera. The results showed that BiP which bound to calreticulin and the BiP associated with assembly-defective phaseolin represented different subsets of the BiP pool (Crofts *et al.*, 1998).

Calreticulin is known to mask BiP epitopes that are not masked by unassembled phaseolin molecules and it has been suggested that it may interact with a different region of the molecule. It is unlikely that calreticulin acts as a co-factor for BiP, as BiP has been associated with either a malfolded protein or with calreticulin, but never with both at the same time (Crofts *et al.*, 1998). It has been proposed that the BiP-calreticulin complex might act as a buffer to modulate the concentration of free BiP in the ER. Any excess BiP not required for the folding of nascent proteins could be recruited into the complex for storage (Crofts & Denecke, 1998). Another possibility is that the BiP-calreticulin complex might be part of a larger structure that involves other chaperones, similar to those detected using cross-linking experiments in mammalian cells (Tatu & Helenius, 1997). The co-

immunoprecipitation of BiP with calreticulin has so far not been demonstrated in mammal cells. Although mammalian calreticulin has been identified in complexes without BiP (Oliver *et al.*, 1997), in plants only a very small proportion of the cellular calreticulin is involved in binding the huge majority of BiP.

2.3 CHAPERONE SELECTION AND INTERACTIONS

Proteins synthesised within the ER lumen interact differently with chaperones, some interacting first with BiP and later with calnexin (Hammond & Helenius, 1994; Kim & Arvan, 1995), whilst others bind calnexin and calreticulin but do not associate with BiP (Braakman *et al.*, 1991; Xu *et al.*, 1998). During the folding of newly synthesised proteins in the ER, BiP has been shown to act sequentially with a member of the HSP90 family GRP94 (Melnick *et al.*, 1994), and to cooperate with PDI (Braakman *et al.*, 1992; Puig & Gilbert 1994) and calreticulin (Tatu & Helenius, 1997).

In a recent study, the maturation of two glycoproteins, E1 and p62, from the Semliki forest virus was analysed in CHO cells to investigate chaperone selection (Molinari & Helenius, 2000). The results from this study suggest that a choice is made between chaperone systems during the translocation of a glycoprotein in the ER. One of the systems comprises BiP and perhaps PDI, whilst the other comprises calnexin, calreticulin and the oxidoreductase enzyme Erp57. The initial binding of one chaperone appears either to delay or prevent binding of the other chaperone.

Mutants of influenza haemagglutinin (IHA) were also made to investigate whether the position of N-linked glycans in the sequence of a glycoprotein correlated with differential chaperone-binding (Molinari & Helenius, 2000). The results indicated that glycoproteins such as p62 and HA, which contain glycans close to the NH_2-terminus, entered the calnexin/calreticulin pathway directly without prior association with BiP. However, proteins such as E1 and the VSV glycoprotein, which are glycosylated closer to the COOH-terminus, initially bound with BiP (Molinari & Helenius, 2000). Using castanospermine, an inhibitor of the calnexin/calreticulin pathway, p62 and IHA could be re-directed to the BiP pathway. This result demonstrated that the presence of N-linked glycans *per se* does not prevent BiP from binding, but rather promotes the association of the alternative chaperones. A number of cellular and viral proteins with N-linked glycans

within the first 50 residues of the NH_2-terminus have all been shown to fold independently of BiP (Molinari & Helenius, 2000).

A large ER-localised multiprotein complex containing a subset of chaperones and folding enzymes - including BiP and PDI - has been identified (Meunier *et al.*, 2002). This subset of ER chaperones formed an ER network capable of binding to unassembled, incompletely folded immunoglobulin heavy chains, rather than existing as free pools that assembled onto the substrate protein. The components of the calnexin/calreticulin system were either not detected in the complex, or only very poorly represented.

3. Guy's 13 Monoclonal antibody and passive immunotherapy

Passive immunisation involves the delivery of pathogen-specific antibodies to a host. Many of the potential side-effects of active immunisation are minimised through local passive immunisation, such as the possibility of eliciting cross-reactive responses. This approach has been proposed as a means of preventing the colonisation of teeth by mutans streptococci and the development of caries (Lehner *et al.*, 1985; Michalek *et al.*, 1987; Filler *et al.*, 1991; Otake *et al.*, 1991).

MAb Guy's 13 is a murine IgG1 class of antibody, and was originally raised against the cell surface adhesin (SA I/II) homologue from *S. sobrinus*. It recognises a common protein epitope shared by all serotypes of mutans streptococci and binds strongly to *S. mutans*, which is the principle causative bacteria in dental caries (Mitchell *et al.*, 1987; Smith & Lehner, 1989). Topical application of MAbs such as Guy's 13, directly onto tooth surfaces, protected against *S. mutans* colonisation and caries in monkeys (Lehner *et al.*, 1985). Similar protection from colonisation was subsequently demonstrated in human volunteers (Ma *et al.*, 1987; Ma *et al.*, 1989). Furthermore, recolonisation by *S. mutans* in human carriers could be completely prevented using MAbs (Ma *et al.*, 1990).

Although Guy's 13 has potential as an anti-caries immunotherapeutic MAb, one significant drawback remains, namely the high cost of generating the large quantities of MAbs required for passive immunotherapy using conventional hybridoma techniques. Recombinant heterologous expression systems may be especially valuable in these circumstances, although an added difficulty in this application, is the requirement for full-

length antibody, rather than antibody fragments which are not protective *in vivo* (Ma *et al.*, 1990).

3.1 MUCOSAL IMMUNITY

Dental caries is the result of a mucosal infection, and therefore represents a valuable model for the study of mucosal immunity by both innate and adaptive defence mechanisms. The innate mechanisms comprise the mucosal barrier, mucins, salivary factors (McGhee & Mestecky, 1990), and various anti-microbial peptides (Weinberg *et al.*, 1998).

Innate immunity not only provides a physical anti-microbial defence, but also determines how pathogens activate the adaptive mucosal immune system and control the nature of the immune response (Bendelac & Fearon, 1997). Adaptive immunity requires the antigens to cross the epithelial layer and reach the underlying organised lymphoid tissue, where antigen presentation and priming of B and T cells can take place. Antibodies play a major role in the protection of all mucosal surfaces, and on average an adult produces and transports into secretions around 10 g each day (McGhee & Mestecky, 1990).

The major salivary immunoglobulin is secretory IgA (SIgA), and in adult whole saliva the relative contributions of SIgA, IgG and IgM are approximately 200, 1, and 1 mg per 1000 ml, respectively (Brandtzaeg *et al.*, 1970). Approximately 60 % of the secretory antibodies are of the SIgA1 subclass, the remainder belonging to the SIgA2 subclass (Delacroix *et al.*, 1982). Antibodies specific for protein antigens are predominantly of the IgA1 subclass, whereas bacterial lipopolysaccharide and carbohydrate antigens lead preferentially to IgA2 antibody production.

As described previously, SIgA antibodies are multivalent, and adapted to cross-link target pathogens in the mucosal environment. SIgA responses can be elicited in saliva by the stimulation of either central lymphoid tissue or local inductive sites of the mucosal immune system. The central inductive sites include gut-associated lymphoid tissue (GALT) and nasal-associated lymphoid tissue (NALT). Stimulation with antigen at these sites results in the sensitisation of specific IgA-committed B cells and their dissemination and homing to remote effector sites, including the salivary glands (Mestecky, 1987).

Compared with SIgA levels in the oral cavity (Challacombe & Lehner, 1976), much smaller amounts of IgM, IgG and monomeric IgA from blood reach the surface of teeth in gingival crevicular fluid (GCF). Levels of these immunoglobulins in GCF are raised in

gingivitis and periodontal disease (Russell *et al.,* 1999), and may play a role in mucosal defence around the oral cavity. Clearly the defence of the oral mucosa is a complex process, supported by a number of versatile and normally highly effective mechanisms.

Mechanism of action of IgA antibodies

Unlike IgG and IgM, IgA is non-inflammatory and does not promote phagocytosis (Kilian *et al.,* 1988). However there are a number of mechanisms whereby SIgA can exert a protective effect; by direct inhibition of microbial adherence to host mucosal epithelial cells (Liljemark *et al.,* 1979), through blocking specific adhesin-receptor interactions, or by interfering with hydrophobic interactions. SIgA may also neutralise microbial toxins and enzymes (Gilbert *et al.,* 1983; Holmgren *et al.,* 1972). SIgA is also capable of viral neutralisation (Russell-Jones *et al.,* 1981) either by direct blocking of receptor binding or through viral aggregation.

SIgA can inhibit microbial penetration through the mucous layer, a process termed immune exclusion (Stokes *et al.,* 1975). The polymeric structure of SIgA enhances its avidity for antigens and promotes microbial aggregation. The consequent formation of complexes facilitates microbial clearance in secretions and reduces the microbial load at the epithelial surface (Neutra *et al.,* 1991). SIgA is also involved in the export of microbes that have already crossed the mucosal barrier. Polymeric IgA in transit to the mucosal surface may encounter microbes or antigens that are already present in the mucosal epithelium or connective tissue, and transport them back out (Kaetzel *et al.,* 1991). This principle has been demonstrated for intracellular neutralisation of Sendai virus in which IgA antibodies interfered with viral replication by binding to newly synthesised viral proteins within infected cells (Mazanec *et al.,* 1992).

4. Production of Monoclonal antibodies

MAbs are conventionally produced by immunising animals (usually rats or mice) with an antigen to stimulate the production of antibody forming plasma cells. These are harvested from the animal's spleen, fused with a myeloma cell line grown in culture, and a single hybrid clone of B cells selected. This "immortal" cell line is called a hybridoma, and the combined output of antibody from a hybridoma is completely homogenous – all the antibody molecules are identical and have the same specificity for antigen (Kohler &

Milstein, 1975).

In the early days of hybridoma technology, hybridoma cells were then injected into the peritoneal cavity of an animal, and MAbs were harvested from ascitic fluid that collected. This technique generates a high yield of antibody, between 1 - 20 mg/ml. However there are disadvantages to using ascitic methods, in particular related to animal welfare concerns. Individual batches of harvested ascitic MAb may vary in quality, and can be contaminated with blood and bioreactive chemokines. There is also a potential risk of contamination by viruses that are pathogenic to humans.

In the last 20 years or so, a range of *in vitro* production systems has been developed. There are three main types of *in vitro* production; static and agitated suspension cultures, membrane-based and matrix-based culture, and high cell density bioreactors. These systems differ in terms of their ease of handling, the yield of antibody, and the maximum achievable antibody titre (reviewed by Hu & Aunins, 1997). However, using a variety of mammalian expression vectors, selection and amplification protocols, CHO cell lines capable of producing MAbs at levels exceeding 1 g/L can now be obtained almost routinely. For the applications of MAbs to expand into additional therapeutic areas, however it was proposed that a 5- to 10-fold increase over these levels may be needed (Trill *et al.*, 1995).

Species that are phylogenetically closer to humans generally have more elements of the glycosylation machinery in common, although there remain differences between the glycosylation characteristics of rodent cell lines and human tissues (Jenkins *et al.*, 1996). Some workers have expressed concerns regarding the quality of IgG MAbs produced *in vitro*, noting that in some cases the glycosylation pattern of antibody generated in bioreactors differs from that *in vivo* (Leibiger *et al.*, 1995). The loss of correct Fv-associated glycosylation can also result in decreased antigen-binding activity (Leibiger *et al.*, 1999).

4.1 BACTERIAL EXPRESSION OF IMMUNOGLOBULIN

Bacterial expression systems offer a convenient means of engineering antibody fragments for a variety of applications (Huston *et al.*, 1988). The host in which most recombinant antibody methods were developed is the bacterium *Escherichia coli*. Growth of bacteria is rapid and inexpensive, and a number of vectors are available for expression and manipulation of cloned genes. DNA can be introduced directly into *E. coli* (transformation)

or by infectious bacteriophage (transfection). Genetic constructions of antibody fragments (such as Fab' and scFv) can be quickly assessed and various selection methods can be applied. It is also easier to scale-up the production of antibody fragments in *E. coli* than in mass culture of mammalian cells such as hybridomas.

Functional antibody fragments can only be assembled correctly in an oxidising environment. In bacterial expression systems this involves the secretion of precursor proteins into the bacterial periplasm. This process mimics the secretion of protein into the lumen of the ER in mammalian antibody-producing cells (Wall & Kuehl, 1983).

However there are several disadvantages associated with bacterial expression systems; recombinant proteins may form inclusion bodies, which necessitates costly *in vitro* refolding. Prokaryotes do not glycosylate heterologous proteins (Letourneur *et al.*, 1995), which effectively excludes the use of *E. coli* for the production of glycoproteins - where the glycan chain is essential for biological activity or efficacy - and bacterial expression systems were not thought to be able to produce full-length antibodies.

Efforts to assemble full-length functional antibodies in bacterial expression systems have been generally unsuccessful. Genes for a murine μ heavy chain and a λ light chain immunoglobulin were inserted into bacterial plasmids containing the *E. coli* trp promoter and ribosome binding site (Boss *et al.*, 1984). The induction of transcription from the trp promoter resulted in accumulation of both light and heavy chains, although both were found as insoluble proteins. However, following extraction and purification of the immunoglobulin containing fractions, antigen-binding activity was detected (Boss *et al.*, 1984).

Recently, a study demonstrated that by optimising the ratio of secreted heavy and light chains, high-level expression and assembly of several full-length humanised IgGs could be achieved in the *E. coli* periplasm (Simmons *et al.*, 2003). In particular, one aglycosylated anti-tissue factor IgG1 antibody (αTF) was thoroughly evaluated, and showed tight binding to antigen and to the neonatal receptor (Simmons *et al.*, 2003). Production of αTF in *E.coli* was scaled up to yield approximately 150 mg/L of assembled antibody, which was then purified using immobilised protein A. If increases in titre can be achieved, this technology may provide a rapid, and potentially inexpensive method for the production of aglycosyl full-length therapeutic antibodies.

4.2 YEAST EXPRESSION

The yeast *Saccharomyces cerevisiae* can synthesise, process and secrete higher eukaryotic proteins (Hitzeman *et al.,* 1983). The addition of a large number of mannose residues to the core oligosaccharide is a common feature of most yeast strains (Herscovics & Orlean, 1993), and has been shown to compromise the efficacy of recombinant proteins, such as the hepatitis B surface antigen (Kniskern *et al.,* 1994). Both yeast and fungi contain high-mannose carbohydrate chains (Jeenes *et al.,* 1991) but are not able to convert these into complex-type structures. Mutant yeast strains, in which N-glycosylation is confined to core oligosaccharide residues with a limited mannose content, have been used to overcome this problem (Kniskern *et al.,* 1994).

Yeast strains that secrete a correctly folded and functional mouse-human chimeric IgG antibody have been produced (Horwitz *et al.,* 1988). Between 50 – 70 % of the heavy chains were associated with light chain, and the chimeric whole antibody exhibited antibody-dependent cellular cytotoxicity and bound to target cancer cells (Horwitz *et al.,* 1988).

The synthesis, processing and secretion of IgM antibody λ light and μ heavy chains in yeast have also been demonstrated (Wood *et al.,* 1985). A significant proportion of the yeast μ heavy chains was N-glycosylated, although the yeast and mammalian μ probably differ in carbohydrate composition. Medium supernatants from cultures of cells transformed with plasmids containing λ or μ inserts, contained λ or μ protein respectively. Functional antibodies were detected in yeast cells that co-expressed both antibody chains *in vivo.* Although antibody activity was recovered from a soluble cell extract prepared from transformed yeast, the efficiency of assembly of functional antibodies was very low (Wood *et al.,* 1985).

The filamentous fungus *Aspergillus niger* has also been used to produce full-length humanised IgG1 (Ward, unpublished data). The antibody was correctly assembled and bound antigen. The secreted level of full-length IgG1 was similar to that found in mammalian systems, and higher than previously reported in microbial systems.

4.3 BACULOVIRUS EXPRESSION IN INSECT CELLS

The baculovirus expression system is capable of expressing biologically active mouse, human, human-mouse hybrid and human-mouse chimeric antibodies of the IgG, IgM and IgA classes as well as antibody fragments in insect cells (reviewed by Potter *et al.*, 1993). It also permits the combinatorial expression of immunoglobulin with other polypeptides, such as J chain, much more rapidly than with stably transfected mammalian lines. The system has the advantage of rapid development, as well as relatively high yields (Haseman & Capra, 1990).

Unlike the reducing environment of bacterial cytoplasm, the insect cytoplasmic environment allows proteins to fold properly, disulphide bonds to form, and post-translational processing, such as N- and O-glycosylation to take place. Ovarian cell lines from *Spodoptera frugiperda* (fall armyworm) cells infected with recombinant baculovirus were used to produce a functional murine IgG heterodimer (Haseman & Capra, 1990), and a murine monoclonal IgG antibody directed against lipoprotein I of *Pseudomonas aeruginosa* (Zu Putlitz *et al.*, 1990). Functional immunoglobulin heterodimers could be produced by coinfecting insect cells with separate heavy and light chain-expressing baculoviruses as well as by infection with a single baculovirus containing both genes under the control of separate promoters (Haseman & Capra, 1990). However in addition to the complete heterodimer, the formation of insoluble immunoglobulin aggregates was observed in the insect cell lysates.

Recombinant human monomeric IgA1 has also been expressed in *S. frugiperda* cells (Carayannopoulos *et al.*, 1994). A baculoviral vector that encodes human J chain was also used to express the J chain in *S. frugiperda* cells. Subsequently, insect cells triply infected with viruses expressing IgA light chain, heavy chain and J chain were able to assemble dimeric IgA. However the assembly was inefficient, as most remained in a monomeric form, which was secreted into the cell supernatant (Carayannopoulos *et al.*, 1994). These data will be discussed in detail in Chapter 5.

Most evidence indicates that the N-glycosylation capabilities of the baculovirus system are limited to producing simple oligomannose-type oligosaccharides (James *et al.*, 1995), and few studies have demonstrated complex N-linked glycans (Davidson *et al.*, 1990). However recently, paucimannosidic oligosaccharides that are not commonly found on mammalian

glycoproteins were identified (Ailor & Betenbaugh, 1999). Immunoblot analysis of baculovirus expressed recombinant IgA1 from cell lysates demonstrated N-glycosylation of heavy chain in the C_H2 region and the C-terminal tail. The type of glycosylation present on the recombinant antibodies was examined by comparing the reactivity of baculoviral IgA1 and serum IgA1 with different lectins. The results were consistent with the presence of N-linked high-mannose oligosaccharides, and O-linked disaccharides on the recombinant IgA1 molecule. Functionally, the recombinant baculoviral IgA monomer was still recognised by several IgA1-specific monoclonal antibodies, and remained susceptible to cleavage by an IgA1-specific protease (Ailor & Betenbaugh, 1999).

The cDNA encoding the N-terminal 589 amino acids of the extracellular domain of the human pIgR has also been inserted into vectors to generate recombinant baculo- and vaccinia viruses (Rindisbacher *et al.*, 1995). After infection of insect and mammalian cells, the resulting truncated protein corresponding to human SC was secreted with high efficiency into serum-free culture medium. The insect cell/baculovirus system yielded up to 50 mg human SC per litre of culture, almost 5 times that produced by the mammalian cells/vaccinia system. Although the M_r of these two forms of recombinant human SC differed, as a result of different glycosylation patterns, both retained the capacity to bind to dIgA purified from hybridoma cells.

One of the major limitations of the baculovirus-insect cell expression system is its inefficiency to process heterologous proteins correctly, and the formation of insoluble aggregates. Consequently, attention has focused on improving the yield of functional proteins using recombinant baculovirus-infected insect cells by supplementing secretory processing proteins which are either absent or limited in supply in the host insect cell. The over-expression of a human HSP70 cytosolic chaperone increases the solubility and secretion of a recombinant IgG protein in insect cells, by enhancing the solubility of the immunoglobulin light chain precursor (Ailor & Betenbaugh, 1998).

5. Transgenic Plant Biotechnology

Products of plant genetic engineering first came to market in the United States, and then more recently in Europe. Examples include a slow-ripening tomato, as well as soy and cotton plants resistant to herbicides and insects (reviewed by Birch, 1997). In the last decade or so it has become clear that plant systems may be especially useful for the expression and production of recombinant molecules. Undoubtedly, one of the major attractions is the potential for growing immunotherapeutic reagents on an agricultural scale, with significantly reduced costs of production.

Modern plant genetic engineering involves the transfer of the desired genes into the plant genome, and subsequently the regeneration of a whole plant from the transformed tissue. The most often used method for transferring genes into plants is *Agrobacterium*-mediated transformation (Zambryski *et al.,* 1989). *Agrobacterium tumefaciens* is a naturally found pathogenic soil bacterium that is able to transfer DNA into a plant's genome. DNA vectors have been designed from the *Agrobacterium* tumour-inducing plasmid (pTi) DNA which carries desired genes into the plant (Zambryski *et al.,* 1989). Tobacco plants can be transformed and regenerated relatively easily using *Agrobacterium*, and as a result they are often used as a model system, along with *Arabidopsis,* potato, tomato, and soy plants (Park *et al.,* 1996; Tinland, 1996).

It had been thought that infection by *Agrobacterium* was limited, by host range restrictions, to dicotyledon and gymnosperm species. However *Agrobacterium*-mediated transformation has also been achieved in monocotyledon species, such as rice and maize (Chan *et al.,* 1993; Ishida *et al.,* 1996; Park *et al.,* 1996). The common monocotyledonous crop plants have proved more amenable to transformation through ballistic micro-projectile bombardment techniques (Christou, 1995). As with *Agrobacterium*, the foreign gene is integrated into the plant genome and traditional breeding techniques can then be employed to generate transgenic seed stocks, for simple and stable storage or distribution.

Alternative methods to express heterologous proteins in plants involve the use of viral vectors. Two main strategies have been employed, the first of which involves foreign gene transcription, in which the foreign gene is expressed as a soluble protein. The second strategy involves the engineering of viral coat proteins in fusion with antigenic peptides or proteins, whilst allowing the continuing assembly and formation of infectious viral particles

that display antigen on their surface. Two of the most widely used plant viruses are tobacco mosaic virus (TMV) [Donson *et al.*, 1991] and cowpea mosaic virus (CPMV) [Lomonossoff & Hamilton, 1999].

5.1 GENE CONSTRUCTS

Foreign genes transferred into plants are chimeric constructs that comprise the gene coding sequence fused to plant functional regulatory signals, the promotor and polyadenylation sequences. The regulatory signals which permit gene expression in plants are often derived from the 19S and 35S transcripts of the cauliflower mosaic virus (CaMV) [Odell *et al.*, 1985], or from *Agrobacterium* genes (Bevan *et al.*, 1983; De Greve *et al.*, 1983; Di Rita & Gelvin, 1987). The choice between constitutive promoters, such as the CaMV 35S or the maize ubiquitin 1 promoter must be evaluated on an empirical basis. It is often desirable to achieve high-level antibody accumulation in the most suitable organs for storage and extraction, normally the leaves and seeds. Seed-specific promoters, such as the glutelin promoter (Goto *et al.*, 1999) may be beneficial in some plants, however constitutive promoters may generate better results in cereals such as rice and wheat (Drakakaki *et al.*, 2000).

Another consideration for vector design is the incorporation of features to permit appropriate targeting of the antibody – for example, deciding whether or not secretion via the endomembrane system is the chosen biosynthetic route. In the first publication that demonstrated the production of antibodies in transgenic plants, transformants from constructs lacking a mouse leader sequence contained very low levels of κ and γ chains, although Southern and Northern blots demonstrated the presence of the transgene (Hiatt *et al.*, 1989). Furthermore, none of the plants that expressed leaderless immunoglobulin chains contained assembled gamma-kappa complexes.

The presence of a mouse leader sequence led to the expression of κ and γ-chains at much higher levels and production of functional antibodies. It was suggested that this might be as a result of enhanced translation of the immunoglobulin messengers or through the increased stability of each protein as a consequence of sub-cellular sequestering or secretion (Hiatt *et al.*, 1989). Increased antibody stability may be achieved by retaining the newly synthesised molecules in the ER. This can be carried out using a 3' KDEL sequence in the vector, which

is translated into a tetrapeptide signal that causes the proteins to be retained in the ER (Conrad & Fiedler, 1998; Napier *et al.*, 1992).

5.2 RECOMBINANT ANTIBODY PRODUCTION IN TRANSGENIC PLANTS

The first full-length antibody produced in plants was an IgG1 antibody (6D4) expressed in tobacco that recognised a synthetic phosphonate ester (P3), and catalysed the hydrolysis of certain carboxylic esters (Hiatt *et al.*, 1989). The γ heavy chain and κ light chain cDNAs were cloned individually into the plant expression vector pMON530 (Rogers *et al.*, 1987). This vector contains plant selectable markers, promoters, *E. coli* and *Agrobacterium* origins of replication and a mouse immunoglobulin signal sequence upstream from the polymerase chain reaction (PCR) insert. Subsequently, transgenic plants were regenerated using these constructs (Hiatt *et al.*, 1989).

Transgenic plants containing the antibody light or heavy chains were cross-pollinated and the seeds harvested (Hiatt *et al.*, 1989). 11 out of 18 of the F_1 offspring expressed both γ and κ chains together whilst Western blotting and ELISAs demonstrated that 95 % of these plants contained assembled functional antibody. The functional activity of the plant-derived antibody was compared with that of the original ascites-derived antibody, by ELISA with a P3-BSA conjugate as antigen. The catalytic activities of both antibodies differed by less than an order of magnitude, and antigen specificity was confirmed by inhibition of P3-BSA binding by free P3, in which the half-minimal inhibition was about 10 mM for both antibodies. A surprisingly high level of accumulation of functional 6D4 antibody was observed in the transgenic plants, greater than 1 % of the total extractable protein (Hiatt *et al.*, 1989).

Coordinate expression of immunoglobulin heavy and light chain genes in a single vector yielded high levels of functional antibodies to a fungal cutinase in the roots of transgenic tobacco (Van Engelen *et al.*, 1994). The heavy and light chain cDNAs were amplified by PCR, fused to a signal sequence for secretion, and expression was under the control of the CaMV 35S and TR2' promoters in a single T-DNA - the chimeric genes were cloned in tandem in a divergent orientation. Western blotting showed assembly to a full-size antibody (Van Engelen *et al.*, 1994), as well as an $F(ab')_2$-like fragment which is probably a result of proteolytic breakdown. The construct with divergent promoters directed accumulation of functional antibodies up to 1.1 % of the total soluble protein (TSP) [Van Engelen *et al.*,

1994].

Currently, a number of MAbs produced in plants are in development. In the United States, clinical trials on an anti-cancer antibody produced in corn have been performed. Transgenic soybeans are also being developed that produce humanised IgG1 antibodies against HSV-2 (Herpes simplex virus-2) [Zeitlin *et al.*, 1998], a disease that infects approximately 20 % of adults in the United States (Fleming *et al.*, 1997).

A humanised IgG1 MAb directed against glycoprotein B of HSV-2 produced in transgenic soybean plants (Zeitlin *et al.*, 1998) was compared with the same antibody expressed in mammalian cell culture (Co *et al.*, 1991) in a number of assays, including diffusion and stability in mucous as well as prevention of vaginal transmission of genital herpes in the mouse. These two MAbs were indistinguishable when examined by SDS-PAGE, and their affinity was almost identical (5.3×10^{-7} M^{-1}) [Zeitlin *et al.*, 1998]. Using a mouse model of vaginal transmission of HSV-2 infection (Whaley *et al.*, 1994), it was demonstrated that vaginal delivery of the plant antibody and the mammalian cell culture-expressed MAb provided similar levels of protection against a vaginal inoculum of HSV-2 (Zeitlin *et al.*, 1998). *In vivo*, the HSV-2-induced cytopathic effect was reduced by 50 % with a MAb dose of 0.2 – 0.3 μg/ml, whereas 100 % protection required a MAb dose of 10 μg/ml. Protection against symptomatic infection required less MAb, 50 % protection being provided by a MAb dose of 0.01 – 0.1 μg/ml, and 100 % protection was provided at a dose of 1 - 10 μg/ml (Zeitlin *et al.*, 1998).

The diffusion of the humanised anti-HSV-2 MAb expressed in soybean plants and mammalian cell culture in human mid-cycle cervical mucous and water was compared (Zeitlin *et al.*, 1998). Neither of the MAbs was slowed in mucous, although it had been suggested that differences in glycosylation might affect the ability of a plant-derived antibody to diffuse in mucous (Saltzman *et al.*, 1994). These findings are important because in order to function effectively on a mucosal surface, antibodies should be able to diffuse freely through mucous and bind to their antigen.

Following exposure to human cervical mucous and semen, both plant and mammalian cell cultured-expressed MAbs had similar neutralising activity *in vitro* (Zeitlin *et al.*, 1998). Following incubation in semen for 25 hours at 37 °C, both MAbs retained 75 % of their neutralisation activity compared with untreated antibody. Subsequently, following

incubation in cervical mucous for 25 hours at 37 °C, the plant antibody retained 100 % of its neutralisation activity (Zeitlin *et al.*, 1998).

5.3 PRODUCTION OF GUY'S 13 MONOCLONAL ANTIBODIES

Guy's 13 IgG was expressed in transgenic tobacco using the same technology as for the 6D4 IgG antibody (Ma *et al.*, 1994). mRNA was purified from the Guy's 13 hybridoma cell line and cDNA prepared. DNA encoding the gamma heavy chains and kappa light chains of Guy's 13 were amplified by PCR using degenerate oligonucleotides, and cloned into pL530, a constitutive plant expression vector derived from pMON530, which contains a mouse immunoglobulin leader sequence upstream of the cloning site (Rogers *et al.*, 1987). The recombinant vector was introduced into *Agrobacterium tumefaciens*.

Two additional forms of a hybrid Guy's 13 heavy chain were constructed using a similar approach. These encoded chimeric heavy chains consisting of Cγ and Cα chain domains. The α-chain sequences were derived from a murine IgA MAb MOPC 315 (Ma *et al.*, 1994). Transgenic plants were regenerated and those that expressed heavy chains were cross-pollinated with those expressing light chains, and the resulting seeds germinated.

Thus three different forms of Guy's 13 MAb were expressed in plants, all of which contain the identical kappa light chain, but have different heavy chains. Plant Guy's 13 IgG1 contains the original gamma heavy chain. Guy's 13 with IgG/IgA hybrid heavy chain consisting of var-γ1-α2-α3 domains was termed plant G1/A, and Guy's 13 with IgG/IgA hybrid heavy chain consisting of var-γ1-γ2-α2-α3 domains was termed plant G2/A (Ma *et al.*, 1994). The latter therefore contains an extra constant region domain when compared with IgG or IgA.

The functionality of the plant antibodies was tested by ELISA, which confirmed the specific recognition of purified SA I/II. The plant antibodies also recognised the native antigen on the surface of *S. mutans* cells. Extracts from transgenic plants that expressed single light or heavy chains were not capable of binding antigen. Competitive inhibition with the three plant antibodies demonstrated recognition of the identical streptococcal epitope to that of the original murine-derived Guy's 13 (Ma *et al.*, 1994). All three plant antibodies caused aggregation of *S. mutans*, which confirmed that the Guy's 13 heavy and light chains assembled into bivalent antigen-binding molecules (Ma *et al.*, 1994).

A total of 10 antibody-producing plants were produced for each form of Guy's 13, in which

the yield of recombinant antibody was approximately 1 % TSP (Ma *et al.*, 1994). This study demonstrated the potential for antibody engineering of the constant region of the antibody, which does not appear to affect the ability of plants to assemble the antibody and suggested that it might be possible to generate a recombinant plant SIgA version of Guy's 13.

The aims of my study were to;

1) analyse immunoglobulin assembly in plant cells and the role of ER-resident chaperones, and to purify plant recombinant immunoglobulins in order to carry out a preliminary analysis of glycosylation,

2) investigate targeting of mammalian membrane proteins to the plant cell membrane, and to determine if functional proteins could be assembled and retained at this site, and

3) to generate a recombinant SIgA in plants, and to analyse its function and make a comparison with its murine IgG counterpart.

CHAPTER 2:

MATERIALS AND METHODS

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CHAPTER 2: MATERIALS AND METHODS

The recipes for all solutions marked * can be found in the Appendix.

2.1 EXPRESSION AND ASSEMBLY OF RECOMBINANT IMMUNOGLOBULIN POLYPEPTIDES IN TRANSGENIC TOBACCO PLANTS

2.1.1 PREPARATION OF CRUDE PLANT EXTRACTS FOR ELISA

Approximately 250 mg leaf tissue from plants between 3 and 4 weeks old was homogenised in 500 µl ice-cold Tris buffered saline (TBS*) containing leupeptin (10 µg ml^{-1}) [Roche Diagnostics, UK], and centrifuged at 14 000 g for 2 minutes. Supernatants were either used immediately or stored in aliquots at – 20 °C. The total soluble protein (TSP) concentration of the plant extracts was measured using the Bradford protein assay (BioRad, UK).

2.1.2 PREPARATION OF ELISA PLATES AND ASSAY CONDITIONS

To screen putative transgenic plants, immunoglobulin heavy and light chain specific capture ELISAs were prepared. An antigen-specific (SA I/II) ELISA was used to detect whole antibody binding to native antigen.

Immobilon™ microtitre plate wells (Nalge, Nunc International, UK) were coated with 50 µl of the appropriate coating solution and left overnight at 4 °C. The coating solutions comprised the following ligands at predetermined optimal concentrations;

a) Purified SA I/II (2 µg ml^{-1}) in TBS was kindly supplied by Dr. Charles Kelly, Guy's Hospital, London,

b) Log phase growth *S. mutans* (NCTC 10449) resuspended in bicarbonate buffer pH 9.8 (*S. mutans* grown in Todd-Hewitt broth (Russell *et al.*, 1980) was also provided by Dr. Charles Kelly,

c) Affinity purified goat anti-mouse κ chain (1 µg ml^{-1}) [TCS Biologicals, UK],

d) Affinity purified goat anti-mouse γ1 chain (0.6 µg ml^{-1}) [SigmaAldrich, UK],

e) Affinity purified goat anti-mouse α chain (1 µg ml^{-1}) [SigmaAldrich, UK].

ELISA plates were then washed three times with TBS containing 0.05 % (v/v) Tween-20 (TBST). To block non-specific binding, 200 µl TBS containing 5 % (w/v) non-fat milk was added to each well, and the plate left for 2 hours at room temperature. The blocking buffer was removed, and the plate used immediately or stored at – 20 °C.

100 µl of sample supernatants was added in duplicate serial two-fold dilutions (in TBS) to the microtitre plate. Non-transformed wild-type plant extracts were used as a negative control. For SA I/II, κ light chain and γ1 heavy chain assays the positive sample control was affinity purified Guy's 13 mouse MAb (50 ng ml^{-1}). For the α heavy chain assay the positive control was an IgA monoclonal antibody (TEPC 21, 2 µg ml^{-1}) [SigmaAldrich, UK]. In all cases incubation was at 37 °C for 2 hours. After washing with TBST, bound immunoglobulin chains were detected with 50 µl of an appropriate conjugated secondary antibody for 2 hours at 37 °C.

The following secondary antibodies were used;

a) Affinity purified goat antiserum to mouse κ chain conjugated with horseradish peroxidase (HRP) [1:1000 dilution, Caltag, USA],

b) Affinity purified goat antiserum to mouse γ1 chain conjugated with either alkaline phosphatase (1:1000, SigmaAldrich, UK),

c) Affinity purified goat antiserum to mouse α chain conjugated with alkaline phosphatase (1:1000, SigmaAldrich, UK),

Alkaline phosphatase-conjugated antibodies were detected with disodium ρ-nitrophenylphosphate (PNPP) [SigmaAldrich, UK]; HRP-conjugated antibodies were detected with 2,2-azino-di-(3-ethyl-benzathiazoline sulfonate) [ABTS] (Roche Diagnostics, UK). Levels of antibody expression were measured by absorbance at 405 nm (A_{405}) or 450 nm (A_{450}) [for PNPP or ABTS respectively].

Expression levels of recombinant proteins were determined by comparison of titration points for plant samples in an antigen specific ELISA, with those of Guy's 13 IgG at known concentrations. For antibody expressing plants, capture was with streptococcal antigen I/II (2 µg/ml). Detection was with HRP-labeled anti-kappa or anti-gamma chain

specific antiserum for IgG, or with rabbit anti-secretory component followed by HRP-labeled anti-rabbit IgG antiserum for secretory antibody.

2.1.3 SDS-PAGE

Crude leaf homogenates (described in section 2.1.1) were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [Laemmli *et al.*, 1970] followed by Western blotting. Samples (10 – 20 µl) were heated at 100 °C for 4 minutes in 2X SDS-PAGE sample buffer* in the presence (reducing conditions) or absence (non-reducing conditions) of 5 % (v/v) β-mercaptoethanol, and loaded onto either 4 % (w/v) or 10 % (w/v) polyacrylamide gels. The 10 % (w/v) acrylamide gels incorporated a stacking gel* comprising 5 % (w/v) acrylamide. Electrophoresis, using a mini gel system (Hoeffer Scientific Instruments, UK), was at 200 V.

2.1.4 WESTERN BLOTTING

For Western blotting, proteins separated on SDS-PAGE gels were transferred onto nitrocellulose membrane (Biotrace NT™ membrane, Pall Gelman Sciences, UK) using a wet vertical transfer system (WB2 blotting apparatus, Anachem, UK), run at 150 V for 30 minutes in transfer buffer*.

After transfer, free binding sites on the nitrocellulose membrane were blocked in TBS containing 5 % (w/v) non-fat milk powder (Marvel, UK) and 0.05 % (v/v) Tween-20 (TBST) for 2 hours at room temperature or overnight at 4 °C. The membrane was then incubated in appropriate primary antisera in TBST for 2 hours at 37 °C.

a) Affinity purified goat antiserum to mouse κ chain (1:1000 dilution; Caltag),

b) Affinity purified goat antiserum to mouse γ1 chain (1:1000; SigmaAldrich, UK),

c) Affinity purified goat antiserum to mouse α chain (1:1000; SigmaAldrich, UK)

d) Affinity purified sheep antiserum to rabbit secretory component (1:500, SigmaAldrich, UK).

The membrane was washed in two changes of TBST for 1 hour at room temperature. The appropriate alkaline phosphatase secondary antibody was then applied for 2 hours at room temperature with gentle shaking;

a) Affinity purified rabbit antiserum to goat IgG conjugated to alkaline phosphatase (1:1000, SigmaAldrich, UK),

b) Affinity purified donkey antiserum to sheep secretory component conjugated to alkaline phosphatase (1:1000, SigmaAldrich, UK)

The membrane was washed a further three times with TBST, and the bound antibody was visualised by incubation with insoluble alkaline phosphatase substrate buffer* containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Promega, UK).

2.1.5 PREPARATION OF PLANT EXTRACTS FOR IMMUNOBLOTTING AND NORTHERN BLOTTING

To ensure the homogenous nature of the cellular material for both protein and RNA extraction, tissue samples were ground in liquid nitrogen and the homogenous powder was split into two aliquots, and either used immediately or stored at – 80 °C.

For protein analyses, the powder from approximately 250 mg leaf or flower tissue from plants between 8 and 9 weeks old, was homogenised in 500 µl ice-cold Tris buffered saline (TBS*) containing leupeptin (10 µg ml^{-1}) [Roche Diagnostics, UK], and centrifuged at 14 000 g for 2 minutes. Supernatants were either used immediately or stored in aliquots at – 20 °C. The total soluble protein (TSP) concentration of the plant extracts was measured using the Bradford protein assay (BioRad, UK).

2.1.6 PREPARATION OF PLANT RNA

Total RNA was extracted from young plants two months after germination, essentially as described by Logemann *et al.*, (1987). Leaf or flower tissue was frozen in liquid nitrogen with a pre-cooled pestle and mortar and rapidly ground to a powder. This was mixed with two volumes of ice-cold guanidine hydrochloride buffer (8 M guanidine hydrochloride, 20 mM MES, 20mM EDTA, 50 mM 2-mercaptoethanol, pH 7). After agitation it was added to one volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1, v/v/v), mixed thoroughly, and centrifuged at 12 000 g for 45 minutes. The aqueous phase was collected, mixed with ethanol (0.7 volumes) and 1 M acetic acid (0.2 volumes), and incubated at –20 ^{0}C for 16 hours. Following centrifugation, the precipitate was washed three times with 3 M sodium acetate, pH 5.2, and then once with

70 % ethanol. The pellet was dissolved in sterile RNAse-free water (SigmaAldrich, UK) containing RNAse free DNAse (Promega, UK), and incubated at 37 ° C for 1 hour, then at 70 ^{0}C for 5 minutes. Assessments of RNA concentration and purity were made using the GeneQuant II RNA/DNA Calculator (Amersham Pharmacia BioTech, UK). RNA samples were stored in aliquots of sterile RNase free water at –70 °C.

2.1.7 ANALYSIS OF BIP AND CALRETICULIN RNA EXPRESSION

Total mRNA, extracted from the leaves and flowers of wild type and transgenic plants, was examined by Northern blotting and hybridisation, using specific DNA probes under conditions that had previously been optimised in a series of preliminary experiments.

2.1.8 PREPARATION OF DNA PROBES FOR NORTHERN BLOTTING

A 2420 base pair DNA fragment (BLP4) from the tobacco homologue of BiP (Denecke *et al.,* 1991) that had been inserted into the *Sma1* site of the plasmid pGEM3Z (Promega, UK) was provided by Dr. Jurgen Denecke, University of Leeds, UK.

E. coli cells (DH5-α GibcoBRL, UK) were transformed with the recombinant pGEM3Z plasmid, essentially as described in section 2.4.4, and miniprep plasmid DNA was prepared using a commercially available kit (Qiagen, UK). A sample of the purified plasmid was digested with *Alu1* and *EcoR1* (Promega, UK) to reveal the DNA insert of expected size, and this linearised DNA was isolated using the QIAquick™ Gel extraction kit, Qiagen, UK. The sequence of the insert was confirmed using a commercial kit employing the di-deoxy chain termination procedure (Sequenase™ Version 2.0, AmershamPharmacia Biotech, UK) – described in section 2.4.5.

Recombinant linearised plasmid DNA (100 ng, dissolved in Tris-EDTA, pH7.4) was boiled for 3 minutes and placed on ice for 2 minutes then centrifuged briefly, before adding to a sterile tube containing a Ready To Go™ d-CTP labeling bead (PharmaciaAmersham Biotech, UK). 5 µl [α- ^{32}P] dCTP (3000 Cimmol^{-1}) and sterile distilled water were added to a total of 50 µl, and mixed gently. The labeling mixture was incubated at 37 °C for 30 minutes. To stop the reaction 5 µl of 0.2 M EDTA (pH 8) was added. Unincorporated nucleotides were removed by passing the mixture through

Sepharose CL-6B (PharmaciaAmersham Biotech, UK). Labelled DNA was stored at –20 °C.

A 1400 base pair DNA fragment from the castor bean calreticulin gene cloned into Bluescript KS^+ (Stratagene, USA) was kindly supplied by Dr. Sean Coughlan, Du Pont Agrochemicals, USA (Coughlan *et al.*, 1997). *E. coli* (DH5-α, GibcoBRL, UK) were transformed with this recombinant plasmid essentially as described in section 2.4.4. Miniprep DNA was prepared using a commercially available kit (Qiagen, UK).

Recombinant Bluescript KS^+ plasmid DNA was digested with *Xho1* and *EcoR1* (Roche Diagnostics, UK) to release the DNA insert of expected size. The calreticulin DNA insert was sequenced as described in section 2.4.5. To prepare the calreticulin probe for use in hybridisations, plasmid DNA was linearised with *Xho1* and incubated with d-CTP Ready To Go™ labeling beads as described above.

2.1.9 NORTHERN BLOTTING OF PLANT RNA

All solutions and equipment contacting RNA were treated by cleaning in RNAeasy solution (MerckEurolab, UK) followed by rinsing in autoclaved 0.1 % (v/v) diethylpyrocarbonate-treated water*. 15 µg of total plant RNA (prepared as described in section 2.1.6) was separated by electrophoresis on a 1.5 % (w/v) agarose gel. For one 250 ml gel, 220 ml of distilled water containing 3.75 g of agarose and 0.1 µg ml^{-1} ethidium bromide was autoclaved for 20 minutes at 120 °C, and the mixture allowed to cool down to 60 °C. 25 ml of 10 X MOPS buffer* preheated to 60 °C, and 7.5 ml 35 % (v/v) formaldehyde were added. The solution was mixed gently and poured into an RNase-free gel casting tray.

For each sample of RNA and RNA markers (SigmaAldrich, UK), the following premix was prepared: 2 µl 10 X MOPS / 3 µl 35 % (v/v) formaldehyde / 10 µl formamide. Up to 5 µl of RNA was added to 15 µl of premix, and the volume brought to 20 µl with water. Immediately prior to loading the samples were heated at 60 °C for 10 minutes, and 5 µl of 5 X RNA loading buffer* was added. The gel was submersed in 1 X MOPS buffer. Samples were run at a constant voltage of 100 V for approximately 2 hours.

The gel was soaked for 30 minutes in 50 mM NaOH / NaH_2PO_4/ 5 mM EDTA, rinsed in RNAse-free water and soaked for a further 45 minutes in 20 X SSPE* to elute the formaldehyde. The gel was viewed under UV light and photographed alongside a transparent ruler. RNA was blotted onto a Hybond N^+ ™ nylon using a transfer pyramid with 20 X SSPE as the transfer buffer for 16 hours. The membrane was air dried for 45 minutes, and the RNA fixed to the membrane by ultra violet (UV) crosslinking (UV Crosslinker, Hoefer Scientific Instruments) for 90 seconds.

2.1.10 HYBRIDISATION

Following Northern blotting, the Hybond N^+ membrane was prehybridised in 50 µl cm^{-2} of prehybridisation buffer* at 42 °C for 3 hours. Labeled probe (prepared as described in section 2.1.8) was denatured by the addition of 1 volume of deionised formamide (SigmaAldrich, UK) and heated for 5 minutes at 90 °C, then kept on ice. The denatured probe was added to the filter, and incubation was for 16 hours at 42 °C. The membrane was washed briefly with 100 ml 0.1 X SSPE / 0.5 % (w/v) SDS at room temperature to remove most of the free probe. 100 ml of the same buffer heated to 42 °C was added to the membrane, with shaking for 30 minutes at 42 °C. One additional wash was usually sufficient to ensure that the membrane background was low. The membrane was washed once with 0.1 X SSPE at room temperature to remove SDS, blotted dry between filter paper and sealed in plastic Saranwrap™, prior to exposure on Biomax™ MS film (Kodak Scientific Imaging, UK) at –70 °C for 72 hours.

2.1.11 IMMUNOBLOT DETECTION OF BIP AND CALRETICULIN PROTEIN

Flower homogenates from 6 wild type plants were pooled and used as a positive control on each protein immunoblot. Leaf extracts from 6 wild type plants were pooled and used as a pooled wild type control.

Crude plant extracts were analysed on 10 % (w/v) polyacrylamide by SDS-PAGE under reducing conditions (Laemmli *et al.*, 1970), followed by Western blotting (as described in section 2.1.4). Polyclonal rabbit antisera to tobacco BiP was kindly donated by Dr. Jurgen Denecke, University of Leeds, UK, and used at 1:5000 dilution. Polyclonal rabbit antisera to tobacco calreticulin was also generously provided by Dr. Denecke, and used at 1:5000 dilution.

The levels of BiP and calreticulin protein in each sample were compared by densitometry using the Syngene GeneGenius™ image analysis software (Synoptics Ltd., Cambridge, UK) to allow relative quantification. Results were expressed relative to the amount of BiP or calreticulin detected in the flower extract present on each Western blot.

2.1.12 PREPARATION OF PROTOPLASTS

To compare the amount of BiP or calreticulin associated with recombinant immunoglobulins in the different plant extracts, it was important that an equal number of plant cells were used in each case. To do this, we prepared equal numbers of plant protoplasts, and carried out immunoprecipitations using either the lysed cell contents, or the protoplast medium supernatant.

Protoplasts were prepared from the leaves of 2-5 week old tobacco leaves. Leaves were surface sterilised by immersion in; 0.5 % (v/v) sodium hypochlorite for 1 minute, 70 % (v/v) ethanol for 30 seconds, 1 % (v/v) sodium hypochlorite for 1 minute, and sterile distilled water for 1 minute, and then air dried in a fume hood. Using a scalpel blade, the surface of the underside of the leaf was cut every 1-2 mm. Leaves were then transferred onto the surface of 7 ml 1 X enzyme digestion buffer* in disposable petri dishes at room temperature. The leaves were placed on the liquid surface without wetting the upper side, then left overnight at 25 °C in the dark.

The digestion buffer was removed, 6 ml of K3 buffer* was added dropwise to the leaves and the dishes shaken gently to release the protoplasts. The suspension was filtered through a sterile 100 μm-mesh nylon filter, previously wetted (bottom side) in K3 buffer. The filtrate was collected in a sterile tube. Protoplasts were transferred into 50 ml Falcon™ tubes (Beckton Dickinson, UK). Tubes were centrifuged for 20 minutes at 60 g using a swing-out rotor without applying the brake. Following centrifugation, vital protoplasts formed a floating layer, whereas dead cells formed a pellet. The vital protoplasts were removed carefully using a wide-bored pipette, and washed twice in four volumes of W5 buffer* by centrifugation at 60 g for 5 minutes. Following a further centrifugation for 10 minutes at 60 g, the pellet was resuspended in 2 ml W5 buffer and cells were counted using a haemocytometer. Cells were centrifuged again, and resuspended in W5 buffer at a concentration of 2×10^6 cells/ml.

2.1.13 IMMUNOPRECIPITATION OF CHAPERONES ASSOCIATED WITH RECOMBINANT IMMUNOGLOBULINS

For immunoprecipitation studies all manipulations were performed on ice or at 4 °C, using ice-cold buffers. 2 x 10^6 protoplasts isolated from transgenic plants were homogenised by adding 4 volumes of ice-cold homogenisation buffer*, supplemented immediately before use with Complete™ anti-protease mix (Roche Diagnostics, UK). Samples were vortexed, centrifuged at 14 000 g for 5 minutes at 4 °C. The insoluble pellet was discarded, and the protoplast homogenate either used immediately or stored at –80 °C.

A fraction of the protoplast homogenate (250 µl) was brought to a volume of 1 ml with NET-gel buffer*. Samples were centrifuged for 4 minutes at 12 000 g, and supernatants transferred to new microtubes, then centrifuged again to remove precipitating material. This supernatant was incubated with rabbit anti-mouse IgG/A/M (Zymed Inc., USA) [1:1000] for 4 hours on ice.

125 µl of a 10 % (v/v) suspension of Protein-A-Sepharose (Amersham Pharmacia Biotech, UK) prepared in NET buffer* was added, and the sample incubated for 2 hours at 4 °C with slow rotation. Protein-A-Sepharose was pelleted by centrifugation for 2 minutes at 12 000 g, the supernatant discarded, and the beads resuspended in 1 ml of NET-gel buffer and centrifuged again - this step was repeated three times. To determine chaperone activity, 4 mM ATP (SigmaAldrich, UK) was included in the NET-gel buffer for three washes. An equal volume of 2X SDS-PAGE loading buffer (between 30 – 40 µl) was added to the beads, mixed well and boiled for 4 minutes. The sample was centrifuged for 2 minutes at 12 000 g at room temperature, and loaded onto 10 % (w/v) SDS-PAGE, prior to Western blot analysis.

2.1.14 DETECTION OF BIP AND CALRETICULIN ASSOCIATED WITH IMMUNOGLOBULIN CHAINS

Chaperone proteins were visualised by Western blotting using either; the polyclonal rabbit anti-BiP antiserum or the polyclonal rabbit anti-calreticulin antiserum, followed by an alkaline phosphatase labeled anti-rabbit IgG antiserum (as described in section 2.1.4).

2.2 N-GLYCOSYLATION OF A RECOMBINANT PLANT VERSION OF GUY'S 13 MAb

2.2.1 PURIFICATION OF RECOMBINANT PLANT IgG

Mature transgenic tobacco plants expressing Guy's 13 MAb were homogenised in a Waring™ blender on ice with 1.5 volumes of extraction buffer (37.5 mM Tris-HCl pH 7.5, 50 mM NaCl, and 10 μg ml^{-1} leupeptin). After centrifugation of the homogenate at 12 000 g for 30 minutes at 4 °C, ammonium sulphate was added to 50 % saturation. After 2 hours at 4 °C, the solution was centrifuged again, and the pellet resuspended in TBS* to 1/5 of the original volume. The final solution was then centrifuged at 25 000 g for 30 minutes at 4 °C. The concentrated extract was filtered through a 0.45 μm filter and loaded onto a HiTrap Protein-G column (Amersham Pharmacia Biotech, UK). The IgG was eluted with 0.1 M glycine-HCl pH 2.7 and neutralised with 1.5 M Tris-HCl pH 9.5. The preparation was loaded onto a goat anti-mouse IgG1 Sepharose column (SigmaAldrich, UK), eluted with glycine-HCl 0.1 M pH 2.5, neutralised with 1.5 M Tris-HCl pH 9.5, dialysed overnight against PBS and passed through a 0.22 μm filter. The protein concentration was estimated by measuring the absorbance at 280 nm.

The purity of the preparations was assessed by SDS-PAGE, essentially as described in section 2.1.3. Plant recombinant Guy's 13 samples were boiled for 4 minutes in 75 mM Tris-HCl (pH 6.8) containing 2 % (w/v) SDS with or without 5 % (v/v) β-mercaptoethanol, then separated on a 10 % (w/v) acrylamide gel. Proteins were visualised by staining acrylamide gels in Coomassie blue (SigmaAldrich, UK) followed by destaining with a solution of 20 % methanol, 10 % acetic acid. Alternatively, the protein bands were transferred to nitrocellulose for Western blotting (as described in section 2.1.4).

2.2.2 PREPARATION OF RECOMBINANT PLANT GUY'S 13 Fab FRAGMENTS

Purified recombinant plant Guy's 13 was incubated with papain (1:100) [SigmaAldrich, UK] in 0.1 M Tris-HCl, pH 7 containing 2 M EDTA and 20 mM cysteine, for 16 hours at 37 °C. The reaction was stopped by incubation with 0.1 M iodoacetamide for 1 hour at 37 °C. Contaminating Fc was removed by incubation with Protein-G Sepharose beads (Amersham Pharmacia Biotech, UK) for 5 hours at 37 °C, and the supernatant was recovered by centrifugation.

2.2.3 ELISA ANALYSIS OF PLANT ANTIBODY AND Fab FRAGMENT BOUND TO SA I/II

ELISA plates were coated with SA I/II at a predetermined optimal concentration (2 μg ml^{-1}), as described in section 2.1.2. Solutions of plant antibody were applied for 2 hours at 37 °C. The plates were washed with TBST, and either anti-mouse κ chain (TCS Biologicals, UK), anti-mouse γ1 chain (SigmaAldrich, UK) or anti-plant xylose antibodies (kindly donated by Dr. Loic Faye, University of Rouen, France) were applied for 2 hours at 37 °C. After further washes, incubations in appropriate alkaline phosphatase-conjugate antisera were performed for 2 hours at 37 °C. Finally, detection was carried out using disodium p-nitrophenylphosphate (Sigma-Aldrich, UK). Absorbance was read at 405 nm.

2.3 TARGETING AND RETENTION OF RECOMBINANT IMMUNOGLOBULIN CHAINS TO PLANT CELL MEMBRANES

2.3.1 IMMUNOGLOBULIN CHAIN DETECTION BY ELISA

Young plant leaves were homogenised in TBS containing leupeptin (10 μg ml^{-1}) as described in section 2.1.1, although 1 % (v/v) Nonidet P40 (NP40) was included in homogenisation buffer as indicated. Following centrifugation at 14 000 g for 2 minutes, the supernatant was added in 3-fold dilutions to microtitre plates that had been coated with either; goat anti-mouse γ_1 antisera (1:1000, SigmaAldrich, UK) or SA I/II (2 μg ml^{-1}), and blocked with 5 % (w/v) non-fat dry milk (Marvel, UK) in TBST buffer. Immunoglobulin chains present in plant homogenates were detected by ELISA as described in section 2.1.2.

2.3.2 ELISA ANALYSIS OF PROTOPLASTS

Protoplasts were isolated from leaf tissue (as described in section 2.1.12) and resuspended at 2×10^6 cells ml^{-1}. For ELISA analysis of protoplasts, supernatants from overnight digestions were also collected and applied to microtitre plates. Protoplast aliquots were also harvested on a sucrose cushion (12 % w/w), resuspended in 1 X enzyme digestion buffer lacking enzymes, and disrupted by sonication. The samples were centrifuged at 50 000 rpm for 45 minutes at 4 °C. Cell pellets were resuspended in TBS

buffer containing leupeptin (10 μg ml^{-1}) and 1 % (v/v) NP40, and applied to microtitre plates as described in section 2.1.2.

2.3.3 IMMUNOFLUORESCENT STAINING OF PROTOPLASTS

Protoplasts were prepared (as described in section 2.1.12), and resuspended at 2 x 10^6 ml^{-1} in W5 buffer. For immunofluorescence studies, protoplasts were not fixed but essentially maintained as live cells. Fluorescein isothiocyanate (FITC) labelled goat anti-mouse kappa chain (1:25, SigmaAldrich, UK), or FITC labelled goat anti-mouse γ1 antiserum (1:25, SigmaAldrich, UK) were added to the protoplasts, and incubated for 3 hours at 25 °C in the dark. Protoplasts were then washed 3 times in 3 volumes of W5 buffer by centrifugation at 60 g for 5 minutes, and viewed by fluorescence microscopy using an Olympus BH2 microscope.

For control samples, protoplasts from a plant transformed with an irrelevant protein were incubated for 3 hours at 25 °C in the dark, with a polyclonal rabbit antibody against *Arabidopsis* plasma membrane proton pump ATPase antisera (1:100), kindly donated by Dr. Mike Sussman (University of Wisconsin, USA). Protoplasts were washed 3 times in W5 buffer as before, prior to incubation with FITC labelled goat anti-rabbit IgG (1:25, SigmaAldrich, UK), for a further 3 hours, and then washed 3 times in W5 buffer.

2.3.4 EXPRESSION OF THE CHEMOKINE RECEPTOR CCR-5

A cDNA clone of human CCR-5 chemokine receptor was kindly donated by Professor Thomas Lehner, Department of Immunobiology, Guy's Hospital, London. Transgenic plants were prepared by Dr. Pascal Drake, Department of Oral Medicine and Pathology, Guy's Hospital, London.

2.3.5 ANALYSIS OF TRANSGENIC PLANTS BY RT-PCR

The expression of the transmembrane domain of the chemokine receptor molecule CCR-5 was investigated by RT-PCR. Total RNA was purified from putative transgenic plants expressing CCR-5 protein, as described in section 2.1.6. RT-PCR was performed using specific CCR-5 oligonucleotides (refer to the Appendix), essentially as described in section 2.4.1.

2.3.6 IMMUNOFLUORESCENCE STUDIES

Protoplasts were prepared from plants (described in section 2.1.12) that were shown to express a product of the expected size in a RT-PCR using specific CCR-5 oligonucleotides. Protoplasts were incubated in W5 buffer containing partially specific monkey antisera (1:100) raised against the human CCR-5 molecule, which was kindly provided by Dr. Lesley Bergmeier, Guy's Hospital, London. The protoplasts were washed, and incubated in W5 buffer containing FITC labelled rabbit anti-monkey IgG antibodies (1:25) [SigmaAldrich, UK], washed and observed under UV microscopy. Protoplasts from non-transformed plant (WT) and from transgenic plants expressing an irrelevant protein were treated in similar fashion, and were examined under UV microscopy.

2.4 EXPRESSION AND ASSEMBLY OF A SECRETORY ANTIBODY IN TRANSGENIC TOBACCO PLANTS

2.4.1 REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

Putative transgenic plants expressing murine J chain were screened for the production of J chain RNA transcript by reverse transcriptase polymerase chain reaction (RT-PCR) with specific J chain oligonucleotides.

RNA can be reverse transcribed into single-stranded cDNA (Mierendorf & Pfeffer, 1987) using Virus Reverse Transcriptase (MMuLV RT) [Promega, UK]. Approximately 2 µg of plant RNA template was used in 20 µl reaction mix comprising; 2 µl Oligo-$p(dT)_{15}$ primer, 2 µl 10 X Reaction buffer (100 mM Tris, 500 mM KCl; pH 8.3), 5 mM $MgCl_2$, 2.5 mM Deoxynucleotide (dNTP) mix, 1 µl RNase Inhibitor (50 U), 0.8 µl MMLV Reverse Transcriptase (20 U), and sterile water as required.

Control reactions were performed in the absence of reverse transcriptase, to confirm that any bands detected by PCR were derived from RNA. Reactions were incubated at 37 °C for 1 hour. Approximately 5 ng of template DNA was amplified in 100 µl reaction mix comprising; 50 pmol oligonucleotide primers, 200 mM dNTP, 10 µl 10 X PCR buffer [100 mM Tris-HCl (pH 9.0), 15 mM $MgCl_2$, 500 mM KCl] with 2.5 U Taq polymerase.

Oligonucleotides were purchased from Oswel (University of Southampton, UK), and were used to amplify DNA encoding specific sequences found in; J chain, γ1 heavy chain, and a constitutive chloroplast ribonucleoprotein (rubisco). Details of the primers can be found in the Appendix.

Amplification was for 30 cycles: at 96 °C for 30 seconds for denaturing, at 55 °C for 60 seconds for annealing, and at 72 °C for 90 seconds for extension. This was followed by a final extension at 72 °C for 10 minutes. Aliquots of the reaction mixture were analysed by agarose gel electrophoresis and used immediately or kept at -20 °C for longer periods.

2.4.2 AGAROSE GEL ELECTROPHORESIS

DNA samples were analysed against a 1 kb or 100 base pair (bp) marker (GibcoBRL, UK) in 1 % agarose gels (Seakem LE agarose gel, Flowgen, UK) in TAE buffer*, following addition of DNA loading buffer*. Electrophoresis was run using the GNA-100 gel electrophoresis apparatus (AmerhsamPharmacia Biotech, UK) at 90 V in TAE buffer. DNA bands were visualised by ethidium bromide staining (0.5 μg ml^{-1} in water) and gels were photographed under UV exposure.

In order to isolate DNA fragments, following staining in ethidium bromide, bands were excised and the DNA recovered using a commercially available gel extraction kit (Qiagen, UK). The samples were resuspended in a volume of 50 μl, and the purity of the DNA was confirmed by gel electrophoresis.

2.4.3 RE-CLONING OF J CHAIN

To confirm the sequence of the J chain construct, we isolated the J chain construct expressed in transgenic plants by RT-PCR and re-cloned into the plasmid vector Bluescript KS^+ (Stratagene, UK), using the restriction enzyme sites *Xho1* and *EcoR1*.

Approximately equal amounts of J chain DNA and Bluescript KS^+ plasmid DNA were used for ligation. The ligation reaction comprised; the insert, vector, 10 U of T4 DNA ligase (GibcoBRL, UK), 1 X ligation buffer [20 mM Tris-HCl, 1 mM EDTA, 5 mM DTT, 60 mM KCl, 50 % (v/v) glycerol, (pH 7.5) AmershamPharmacia Biotech, UK], in

a total volume of 20 µl. Ligations were incubated at room temperature overnight, and then de-salted by passing through Sepharose CL-6B.

2.4.4 TRANSFORMATION

1 µl of recombinant Bluescript KS^+ vector was incubated with 20 µl *E.coli* cells (DH5-α, Gibco BRL, UK) on ice for 30 minutes. The mixture was incubated at 42 °C for 45 seconds, then placed on ice for 2 minutes. 500 µl sterile SOC medium* was added, and incubated at 37 °C for 2 hours. Cells were plated onto solid Luria Bertani (LB) medium* containing ampicillin (100 µg ml^{-1}), and coated with 40 µl 2 % X-Gal solution* and 4 µl 1M IPTG solution*, and incubated at 37 °C overnight. White colonies resistant to the antibiotic were picked and grown in LB medium containing 100 µg ml^{-1} ampicillin at 37 °C overnight. Plasmid DNA was purified using a commercially available kit (Qiagen, UK) and stored at – 20 °C.

An aliquot of the purified plasmid DNA was digested with *Xho1* and *EcoR1* in accordance with supplier's instructions. Protocols for the use of these enzymes can be found in Sambrook *et al.*, (1989). Samples of digested plasmid, and undigested plasmid (as a control) were assessed by agarose electrophoresis.

2.4.5 DNA SEQUENCE ANALYSIS

Purified plasmid DNA prepared from J chain expressing transgenic plants was sequenced by the di-deoxy chain termination procedure, using T3 and T7 sequencing primers (AmershamPharmacia Biotech, UK) - details in the Appendix - and a commercially available kit (Sequenase™ Version 2.0, Amersham Pharmacia Biotech, UK).

1 – 5 µg double stranded DNA (ds DNA) in a volume of 5 µl was denatured by incubation with 2 µl of NaOH (2N) for 10 minutes at room temperature. DNA was recovered by spin column chromatography using Sepharose CL-6B into a tube containing 1 µl of primer at 5 pmolμl^{-1} and 2 µl of 5 X buffer (200 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 250 mM NaCl). Labeling mix (5.5 µl) for each reaction was comprised of 1 µl 0.1 M DTT, 2 µl of labeling mix buffer (7.5 µM dGTP, 7.5 µM dCTP, 7.5 µM dTTP; diluted 1/5 in water), 0.5 µl (0.185 MBq) of [^{35}S]-dATP, and 2 µl of T7 DNA sequenase (13 Uμl^{-1}) diluted 1/8 in diluting buffer [10 mM Tris-HCl (pH 7.5),

5 mM DTT, 0.5 mg ml^{-1} Bovine Serum Albumin (BSA)] to the DNA. 3.5 µl of the mix was then added to four tubes containing 2.5 µl of ddNTPS (A-C-G-T). Samples were incubated at 37 °C for 5 minutes. Reactions were stopped by adding 4 µl of stop buffer (95 % formamide, 20 mM EDTA, 0.05 % bromophenol blue, 0.05 % xylene cyanol FF). Samples were either analysed immediately or stored at –20 °C.

Samples to be analysed were heated for 2 minutes at 90 °C, immediately put on ice and aliquots (3 µl) were applied to a sequencing gel. The gel was run with parameters set at 2000 V, 50 – 60 mA and 50 – 60 W. Electrophoresis was for 30 minutes to 6 hours depending on the distance from the primers that had to be sequenced. Gels were then transferred to a fixing solution for 30 minutes before being dried on a heat vacuum device and exposed to Kodak Biomax MR-1 X-ray film. The film was developed and the sequence was read manually.

CHAPTER 3:

RESULTS I

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CHAPTER 3: RESULTS I

Expression and assembly of recombinant immunoglobulin polypeptides in transgenic tobacco plants

As described in Chapter 1, a number of transgenic plant lines have previously been prepared, that were studied in this thesis. In this Chapter these plants were utilised to investigate specific events in the plant secretory pathway, namely protein folding and glycosylation. The ability of plants to perform these functions represents two of the key benefits of this eukaryotic heterologous protein expression system.

The original observation (Hiatt *et al.*, 1989) that functional immunoglobulins were correctly assembled and expressed at high levels in plants, even though there are no homologous proteins in plants, suggested that the mechanisms of protein assembly in plants and mammals are broadly similar. Within the endoplasmic reticulum (ER) of mammalian cells, a series of chaperones and enzymes are involved in protein assembly, of which the best characterised include the immunoglobulin binding protein (BiP) and calreticulin. Homologues of these molecules have been identified in plants, and we wanted to confirm their involvement in antibody assembly in plants.

Within the ER and further along the secretory pathway, N-linked glycosylation is an important post-translational modification. As discussed earlier, the addition of glycans to a protein not only influences protein folding, but also the susceptibility to proteolytic degradation, protein function and immunogenicity. Therefore, we carried out a preliminary analysis of N-linked glycosylation on plant derived IgG.

Overall the aims were;

1) to identify transgenic plants expressing either immunoglobulin heavy or light chains, or functional assembled IgG from seed stocks and characterise the expression levels of each recombinant protein.

2) to determine if plant BiP and calreticulin participate in transgenic plants, in the folding of recombinant immunoglobulin heavy and light chains and the assembly of an IgG monoclonal antibody.

3) to purify recombinant IgG from plants and perform preliminary glycosylation analysis.

3.1 Identification of transgenic plants and comparison of recombinant protein expression levels.

Stable homozygous seed stocks were used to generate the plants used in this study. 6 plants representing each line were selected and the accumulation of transgenic gene product (none, kappa chain, gamma chain or both chains) was examined. Kappa and gamma chain capture ELISAs were performed to detect the presence of these chains, an assay for co-expression of kappa and gamma chains utilised streptococcal antigen on the solid phase. This latter assay confirms and measures the assembly of functional antibody.

The ELISAs confirmed recombinant protein accumulation and demonstrated that the titration curves for each of the 6 transgenic plants in each group were very similar, with no significant differences observed between plants in the same group (Figure 3.1), although there were differences between groups. In all the assays, supernatant from a Guy's 13 hybridoma cell culture was used as positive control, and an extract from a wild-type non-transformed tobacco plant was used as the negative control.

The relative accumulation levels for each group were calculated from ELISA results by comparison with known Ig standards, and expressed as a percentage of total soluble protein:

Construct	Percentage Mean +/- standard deviation
κ	0.007 +/- 0.001
γ	0.207 +/- 0.023
IgG	1.270 +/- 0.210

Table 1: Accumulation levels of recombinant immunoglobulins in transgenic plants.

Thus, γ chain accumulation was approximately 30 times higher than κ chain accumulation, which is consistent with previous findings (Hiatt *et al.*, 1989). The level of accumulation of functional IgG antibodies was higher still, with greater than 1.2 % of total extractable protein found to be functional IgG antibody.

3.2 BiP

3.2.1 Detection of BiP RNA

Northern blot analysis of transgenic plants using a BiP DNA probe is shown in Figure 3.2. Flower and leaf tissue from six transgenic plants representing each construct were used and results from 2 plants in each group are shown. A transcript of the expected size for BiP was found in all plant lines in both flowers (Panel A) and leaves (Panel B). Overall, the BiP transcript was more prominent in flower samples as expected (Denecke *et al.*, 1991). Between lines, the levels of hybridising transcripts varied, with a pattern that was consistent between flower and leaf samples. BiP transcript was highest in those plants expressing the heavy chain alone. Low transcript levels were detected in the wild type plants or those expressing light chain alone, and an intermediate level of transcript expression was found in plants expressing assembled IgG.

The results suggest that BiP expression may be increased when cells are actively secreting proteins such as recombinant immunoglobulin heavy chain or assembled immunoglobulin. However, BiP mRNA levels do not correlate with overall recombinant protein expression levels, as although IgG plants express approximately 6-fold more protein than the respective heavy chain plants, the mRNA expression level is visibly less.

3.2.2 Detection of total BiP protein in transgenic plants

Western blotting was used to detect BiP protein in crude leaf extracts from transgenic plants. A representative immunoblot is shown in Figure 3.3, in which extracts from two wild type plants, and two plants each expressing: unassembled heavy chains, unassembled light chains, or assembled immunoglobulin were analysed. Equal amounts of leaf tissue were used to prepare the samples, and detection was with an anti-tobacco BiP antiserum, kindly provided by Dr. Jurgen Denecke.

The crude extract from the flower of a wild type plant was used as a positive control to demonstrate the expected position of BiP (M_r 75,000) on Western blot. The flower extract was used as a marker solely because BiP protein levels are normally higher in flowers (Denecke *et al.*, 1991). A single immuno-reactive band of the expected size was detected, and no cross-reactive bands were found. Using the same mass of starting leaf material, the lowest level of total BiP was detected in leaf extracts from wild type plants, and no difference was observed in plants that expressed unassembled κ light chain. The highest

levels of BiP protein were detected in leaf extracts from plants expressing unassembled gamma heavy chains, and those that expressed assembled IgG.

The same BiP specific immunoblots were performed using all 6 plants for each transgene previously assayed by ELISA for recombinant protein expression (section 3.1) [data not shown]. The relative total levels of BiP were then compared by densitometric analysis of the immunoblots. For standardisation purposes, the same pooled sample derived from flower tissue was used in each blot, and arbitrarily measured as 100 % for the purposes of densitometric measurements. Furthermore, the same pooled sample from wild type plant leaf tissues was used throughout.

Individually, BiP protein expression in each group of 6 plants was fairly uniform. The wild type plant leaf samples yielded a mean densitometry reading approximately 55 % that of the flower sample (Figure 3.4). In leaf tissue from transgenic plants expressing the kappa light chain, the gamma heavy chain, and IgG, mean total BiP protein expression was higher than that in wild-type plants. However, only the differences between γ and WT groups, and between IgG and WT groups reached significance ($F=6.41$, $p=0.003$ ANOVA). In the γ and IgG groups, BiP protein expression was approximately double that of wild type plants. However, no significant difference was observed between γ and IgG plants.

3.2.3 Detection of BiP protein associated with immunoglobulin chains

To investigate the specific association of BiP with recombinant immunoglobulin chains we prepared protoplasts from wild type and transgenic plants (expressing κ light chain, γ heavy chain and full-length IgG) and looked for co-immunoprecipitation of BiP with the recombinant proteins.

Equal numbers of protoplasts were prepared from each transgenic or control plant, lysed, and the lysates were immunoprecipitated (described in Materials and Methods) using an antisera to murine light and heavy chains. Following SDS gel-electrophoresis and blotting onto nitrocellulose, immunodetection was with an anti-BiP antiserum and results from one series of experiments are shown in Figure 3.5 panel A. Co-precipitating BiP was clearly recovered from transgenic plants expressing only heavy chain (γ). A smaller amount of BiP was co-precipitated from plants that produced assembled immunoglobulin (IgG). A faint BiP band was detected from plants expressing light chain (κ) alone. No BiP was precipitated from wild type plants that did not express recombinant immunoglobulin

chains, and there were no cross reactive bands from this preparation or from a control reaction containing only the protoplast lysis buffer.

It had already been demonstrated in a collaborative study that the majority of IgG is secreted from protoplasts (Frigerio *et al.*, 2000). To demonstrate that the BiP interaction with immunoglobulin chains was an intracellular phenomenon, the immunoprecipitation experiment was also carried out using protoplast lysates in comparison with protoplast culture medium (Figure 3.5B). The anti-BiP antiserum did not cross-react with Guy's 13 IgG. BiP was detected in immunoprecipitates from IgG transgenic protoplasts, but not from the protoplast medium, indicating that BiP is not secreted as a complex with immunoglobulin.

To confirm that co-precipitation of BiP reflected real chaperone action, we tested whether the interaction of BiP with immunoglobulin chains was sensitive to ATP. For these experiments, protoplasts were prepared from transgenic plants expressing only the Ig heavy chain. The cells were lysed as before and cell homogenates were immunoselected with either anti-BiP or anti-mouse IgG antisera. The results are shown in Figure 3.6. With anti-BiP immunoprecipitation, a BiP band is detected in samples with or without addition of ATP at equivalent levels. A strongly cross-reactive polypeptide which is approximately the same size as the γ chain (M_r 50K) is also detected, but this is most likely to represent the heavy chain of the rabbit anti-BiP immunoglobulin used for immunoprecipitation. When immunoprecipitation was performed with a Goat anti-Mouse IgG antiserum, the BiP reactive band is detectable in the absence of ATP (right hand panel). However, the intensity of this band is diminished upon incubation with ATP, suggesting a ligand-chaperone relationship between the BiP and Ig heavy chain.

3.3 Calreticulin

Calreticulin, like BiP, is a highly conserved ER resident chaperone (Denecke *et al.*, 1995). It is the major calcium binding protein in the ER (Michalak *et al.*, 1992) and it has been shown to form an abundant complex with BiP (Crofts *et al.*, 1998). The role of calreticulin as a chaperone was demonstrated in the folding and assembly of major histocompatability complex (MHC) class I molecules (Sadasivan *et al.*, 1996), and it is known to bind specifically to newly synthesised glycosylated proteins (Otteken & Moss, 1996). However,

in plasma cells, no role has yet been reported for calreticulin in the folding and assembly of immunoglobulins.

3.3.1 Detection of calreticulin RNA

Using the same plants described for the BiP studies, RNA was extracted from the leaves and flowers of each of the 6 plants representative of each construct, and used in Northern hybridisations with a calreticulin specific DNA probe. The results from two plants (per construct) are shown in Figure 3.7.

A transcript of the expected size (1.4 kb) for calreticulin was found in each of the plant lines examined. The results mirrored those for BiP in that the levels of calreticulin transcript were highest in plants expressing the heavy chain alone (γ), and the lowest levels were found in wild type plants or those expressing light chain alone (κ). An intermediate level of transcript expression was found in plants expressing assembled IgG. Similar results were found in flower and leaf tissues.

3.3.2 Detection of total calreticulin protein in transgenic plants

Western blotting was used to detect total calreticulin protein in crude leaf extracts prepared from wild type and transgenic plants. As with BiP (Section 3.2.2), 6 plants per construct were used, and the western immunoblot bands were analysed by densitometry, and compared against the standardised flower extract (100 %).

The pattern of total calreticulin expression in the different plants was similar to that found for BiP. The lowest levels were detected in wild-type leaf extracts and plants expressing kappa chain (κ) alone. Higher levels of calreticulin were detected in plants expressing assembled IgG and the highest expression levels were in plants expressing unassembled immunoglobulin heavy chains (γ). In this case, the differences between both WT and Kappa groups compared with the Gamma group were statistically significant ($F=6.65$, $p=0.003$ ANOVA).

3.3.3 Detection of calreticulin protein associated with immunoglobulin chains

In contrast to BiP, it was not possible to detect calreticulin in association with recombinant immunoglobulin chains (Figure 3.9). Protoplasts were prepared from non-transformed (WT) and transformed (γ and IgG) plants. Lysed cell extracts were immunoprecipitated with antiserum to murine IgG (heavy and light chains), and after SDS-PAGE and

immunoblotting, detection was with either anti-calreticulin (A) or anti-BiP (B) antisera. No co-precipitating bands of the expected size for calreticulin were detected from any plant (Panel A), as compared with calreticulin present in a WT flower extract. However, as shown previously, co-precipitating BiP was detected from the same heavy chain γ plant sample but not WT (Panel B).

3.4 N-glycosylation of a recombinant plant version of Guy's 13 MAb

Post-translational modification of proteins by glycosylation is a characteristic of all higher eukaryotes, and plant proteins contain both N-linked and O-linked glycans. Most clinically significant mammalian proteins including antibodies, are glycosylated, and their glycosylation can affect their physiochemical properties. Indeed, many of the properties of antibodies are dependent on their glycosylation; such as immunogenicity, half-life *in vivo*, sensitivity to proteases, normal secretion, and correct assembly (Crottet & Corthesy, 1998; Taylor & Wall, 1988).

Native complex glycans in plant proteins can be heterogenous, although they are usually smaller than mammalian complex glycans and contain different terminal sugar residues (Lerouge & Faye, 1996). Complex glycans in plants, as in insects and yeast, do not contain sialic acid residues, and may contain xylose and/or α(1,3)-fucose residues instead of α(1,6)-fucose residues (Sturm *et al.*, 1987). N-acetyl neuraminic acid is a prevalent terminal residue in mammals but has not been shown in plants. The aim in this study was to perform a preliminary analysis of antibody glycosylation in plants, in anticipation of a definitive structural analysis.

3.4.1 Purification of recombinant plant IgG

To facilitate N-glycosylation analysis it was necessary to purify the recombinant plant version of Guy's 13 from mature transgenic plants. Plant proteases and secondary metabolites can complicate the purification of compounds from plant tissues, but relatively few simple steps were sufficient to purify the recombinant plant immunoglobulins (described in Materials and Methods).

The IgG was purified from mature tobacco plants, essentially by ammonium sulphate precipitation and protein G affinity chromatography. The yield of IgG antibody over 5 separate purifications each processing approximately 1Kg fresh weight plant tissue was between 1 - 2.5 mg kg^{-1} fresh weight of plant leaves.

The purity of the preparations was assessed by SDS-PAGE; plant recombinant Guy's 13 samples were separated under non-reducing conditions using SDS-PAGE in 7.5 % (w/v) acrylamide, and visualised by staining in Coomassie blue (Figure 3.10). Samples were taken at various points along the purification process. The crude plant extract (lane 1) shows no evidence for an IgG band of the expected molecular size, suggesting that the expression levels are below the limit of detection. Ammonium sulphate precipitation (lanes 2 and 3) concentrates plant proteins. Following purification on protein G beads, only 4 major bands were eluted (lane 4), the highest band migrates at the expected rate for this IgG. Three other protein bands observed may represent contaminants or degradation products of IgG. The lower two of these bands were removed by the anti-Mouse IgG sepharose affinity chromatography step (lanes 5-7), leaving a purified eluate consisting of primarily of IgG. These fractions were also monitored by antigen specific ELISA to confirm that the purified protein retained antigen binding activity (Figure 3.11). Guy's 13 hybridoma cell culture supernatant and extraction buffer were used as positive and negative controls respectively. Antigen binding was observed in the starting material (crude plant extract), as well as the eluates from the protein G and anti-IgG affinity chromatography steps. There was no significant loss of activity between these latter two steps, even though the SDS-PAGE indicated removal of two major contaminating bands.

3.4.2 Distribution of N-glycans on recombinant plant Guy's 13 MAb

The predicted amino acid sequence of the MAb Guy's 13 heavy chain had revealed two potential sites for N-linked glycosylation (unpublished data). These are the common conserved site in the hinge region of the Fc fragment and a site on Asn-74 located in the variable domain. Fab fragments of the murine Guy's 13 IgG were prepared by papain digestion, and through a collaboration with Dr. Loic Faye at University of Rouen, we have shown that both sites are glycosylated in the mouse derived version of the antibody. The distribution of plant N-glycans on the two potential N-glycosylation sites of the plant version of Guy's 13 was therefore investigated by western blotting and ELISA analysis. The purified plant IgG was digested by papain. Coomassie blue staining of the digested sample demonstrated that the digestion had progressed to completion (Figure 3.12). Samples were run under reducing and non-reducing conditions. Under reducing conditions (left panel), the control IgG degrades into heavy (approx. M_r 55K) and light (approx. M_r 25K). The digested Fab preparation resolves into light and heavy chain (Fd) fragments, the

latter having an approx. M_r of 30K. Under non-reducing conditions (right panel), intact IgG migrates with an approx. M_r of 180K. There is no evidence of intact IgG in the two papain digested samples, the largest visible bands running between M_r 40-50K. These are likely to represent the Fab fragment and Fc fragment. Smaller fragments are likely to represent further breakdown products of the Fc fragment, as papain is also known to cleave at two further positions in the Fc.

The immunoglobulin fragments were separated by SDS-PAGE under non-reducing conditions and immunoblotted with anti-heavy and anti-light chains, as well as with plant glycan (β-(1,2)-xylose)-specific antibodies, kindly supplied by Dr. L. Faye (Figure 3.13 A). All three detecting antibodies bound to intact IgG, thereby confirming that the plant derived IgG was glycosylated (lanes 1). A band at approximate M_r 48K was recognised by both the anti-heavy and anti-light chain antisera, suggesting that this represents the Fab fragment. This band was also recognised by the anti-plant β-(1,2)-xylose antiserum. A smaller fragment (approx. M_r 25-30K) was also recognised by both the anti-heavy and anti- plant β-(1,2)-xylose antisera, but not the anti-light chain antiserum (lanes 2). This is likely to represent an Fc fragment that includes the glycosylation site at the Fc hinge region. The specificity of the anti- plant β-(1,2)-xylose antiserum was confirmed by the absence of binding to murine derived IgG and Fab (lanes 3 and 4).

These results were confirmed in a functional ELISA. As shown in Figure 3.13B, the full-length plant version of Guy's 13 bound to SA I/II, and was recognised by anti-heavy chain (anti-Fc fragment), anti-light chain, and anti-xylose antibodies (Columns 1). Fab fragments produced by papain digestion of the plant antibody also bound SA I/II, and were still recognised by anti-light chain and anti-xylose antibodies but not by anti-heavy chain antibodies (Columns 2). The detection of plant N-glycans on the Fab fragment was confirmed after protein G absorption of the papain-digested material to remove any contaminating intact IgG (Columns 3).

Overall the results demonstrate that Guy's 13 IgG antibody is glycosylated in plants in a manner similar to that found in the murine derived version. The data confirms that not only is the hinge region glycosylation site utilised, but also the N-glycosylation site within the Fab fragment (i.e. Asn74 on the heavy chain). As described in the following Discussion section, this work led to the collaboration with the group of Dr. Loic Faye at the University

of Rouen, in which we have performed a complete structural analysis of the glycoforms associated with both the plant and the murine version of Guy's 13 antibody.

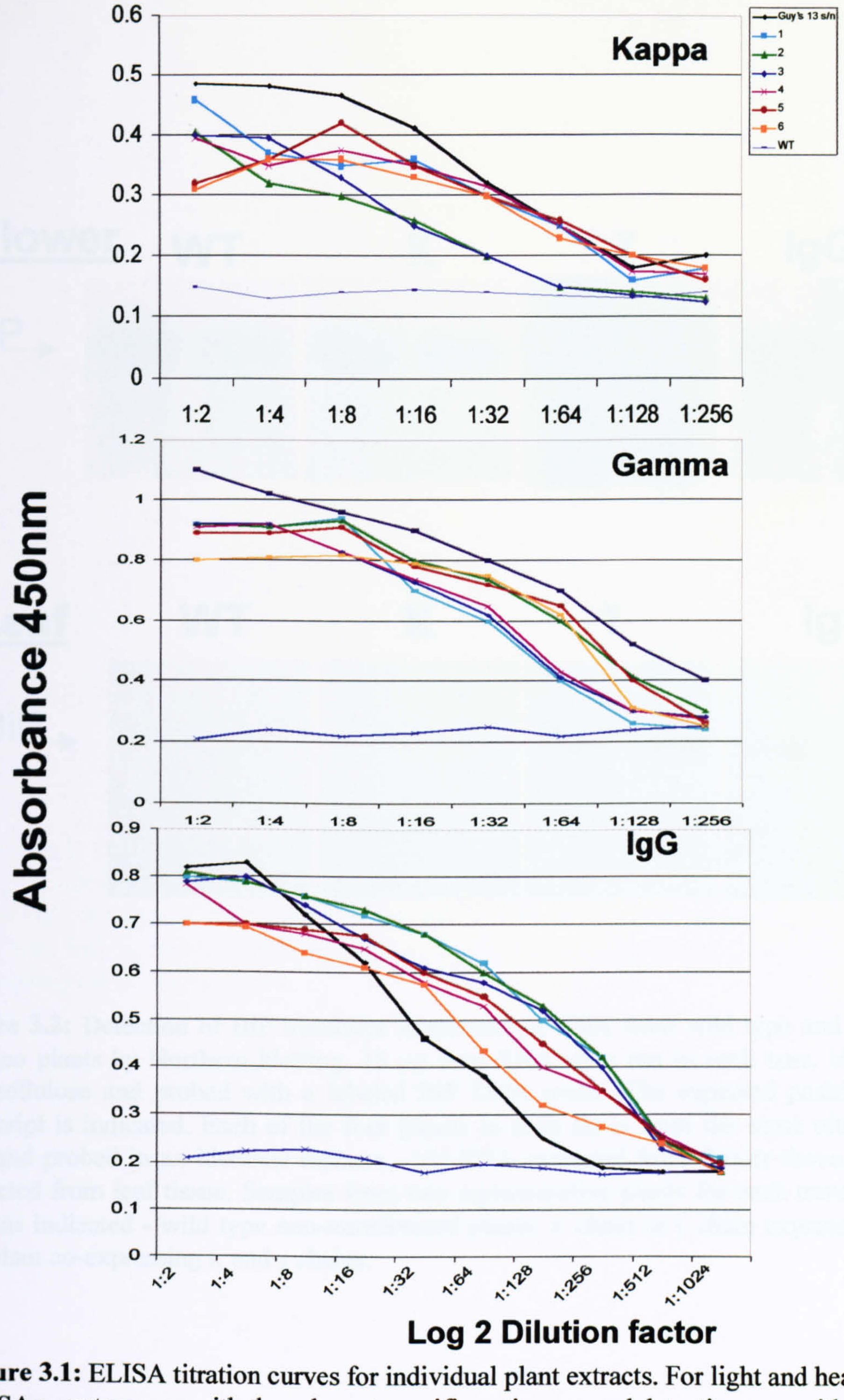


Figure 3.1: ELISA titration curves for individual plant extracts. For light and heavy chain ELISAs, capture was with the relevant specific antiserum and detection was with a horseradish peroxidase labeled antiserum. For IgG assay, capture was with streptococcal antigen I/II at 2 μg ml^{-1} and detection was with a horseradish peroxidase labeled anti-IgG antiserum. Controls were Guy's 13 IgG hybridoma cell culture supernatant and an extract from a wild type non-transformed plant. Results are the mean absorbance from duplicate wells.

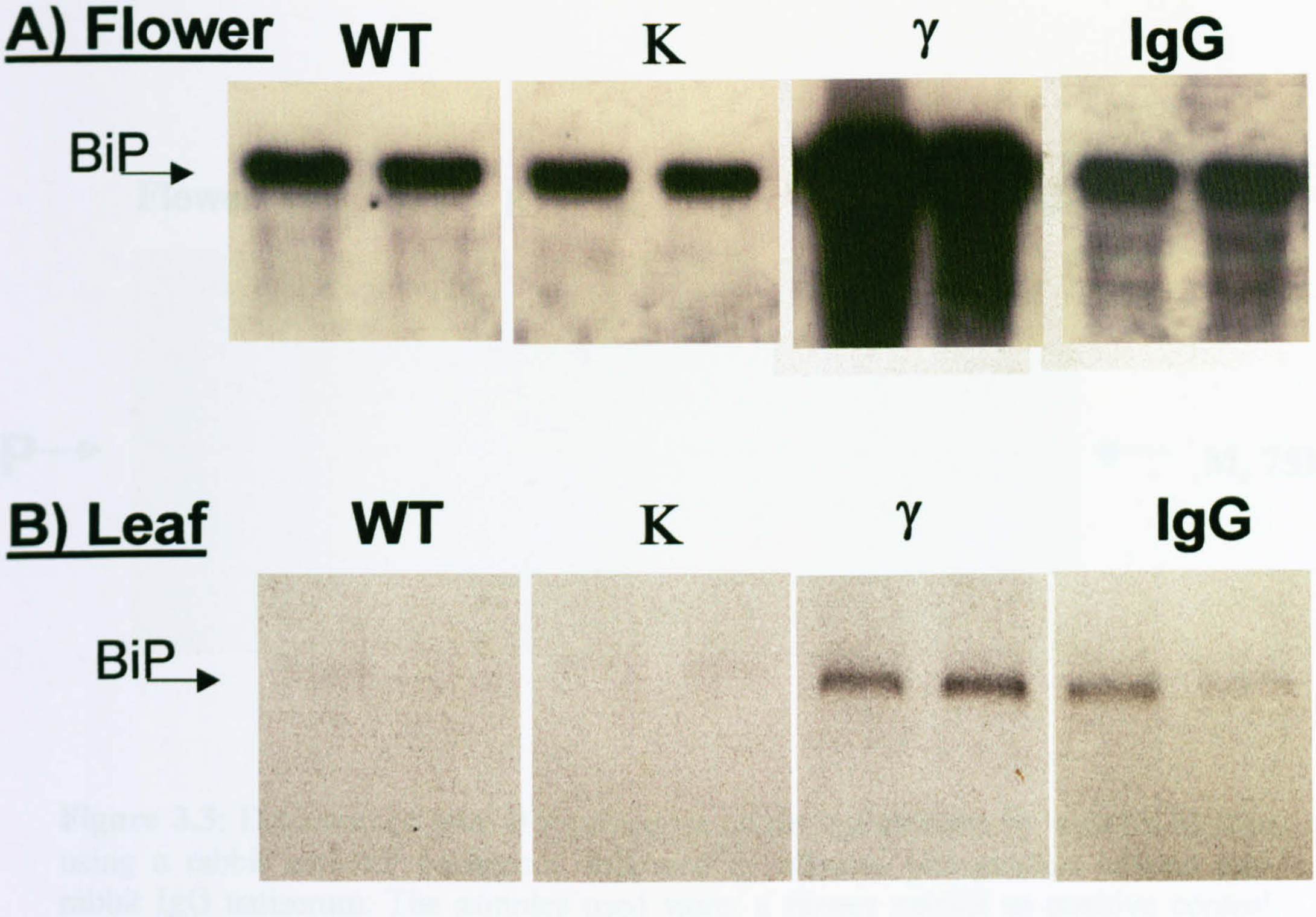


Figure 3.2: Detection of BiP transcript in extracts of RNA from wild type and transgenic tobacco plants by Northern blotting. 15 μg total RNA were run in each lane, blotted onto nitrocellulose and probed with a labeled BiP DNA probe. The expected position of BiP transcript is indicated. Each of the four panels in each set is from the same nitrocellulose blot and probed in an identical manner. (A) RNA extracted from flower tissue, (B) RNA extracted from leaf tissue. Samples from two representative plants for each transgene were used as indicated - wild type non-transformed plants, κ chain or γ chain expressing plants, and plant co-expressing κ and γ chains.

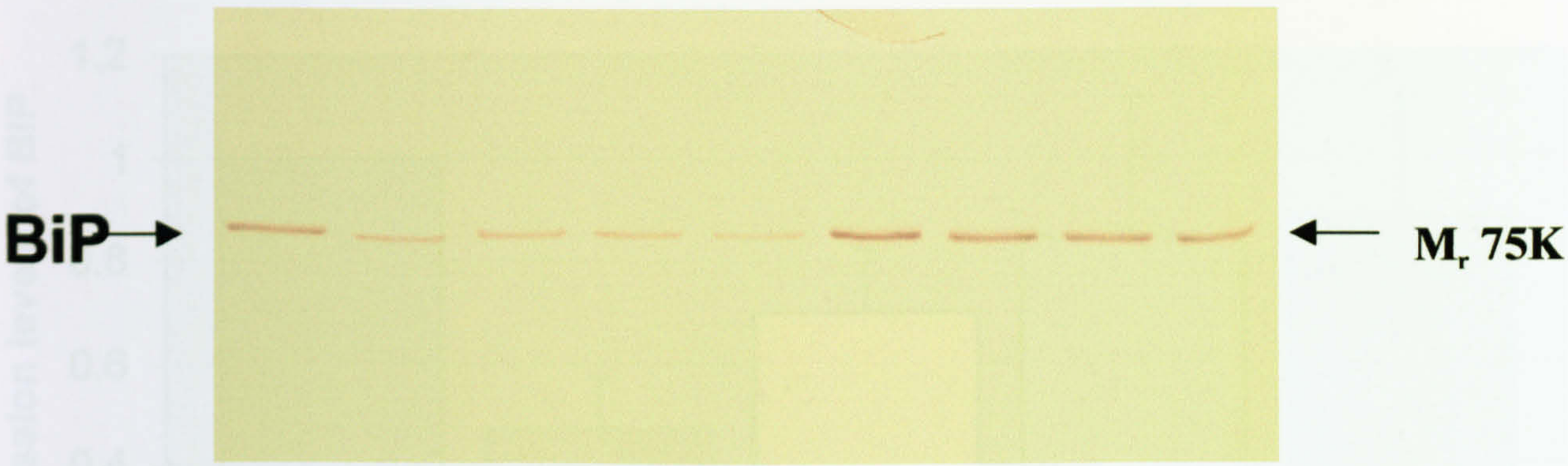


Figure 3.3: Detection of total BiP protein in crude leaf extracts by western blotting using a rabbit anti-BiP antiserum, followed by alkaline phosphatase labeled anti-rabbit IgG antiserum. The samples used were: a flower extract as positive control, wild type leaf extracts, κ chain expressing plants, γ chain expressing plants, and IgG expressing plants, as indicated.

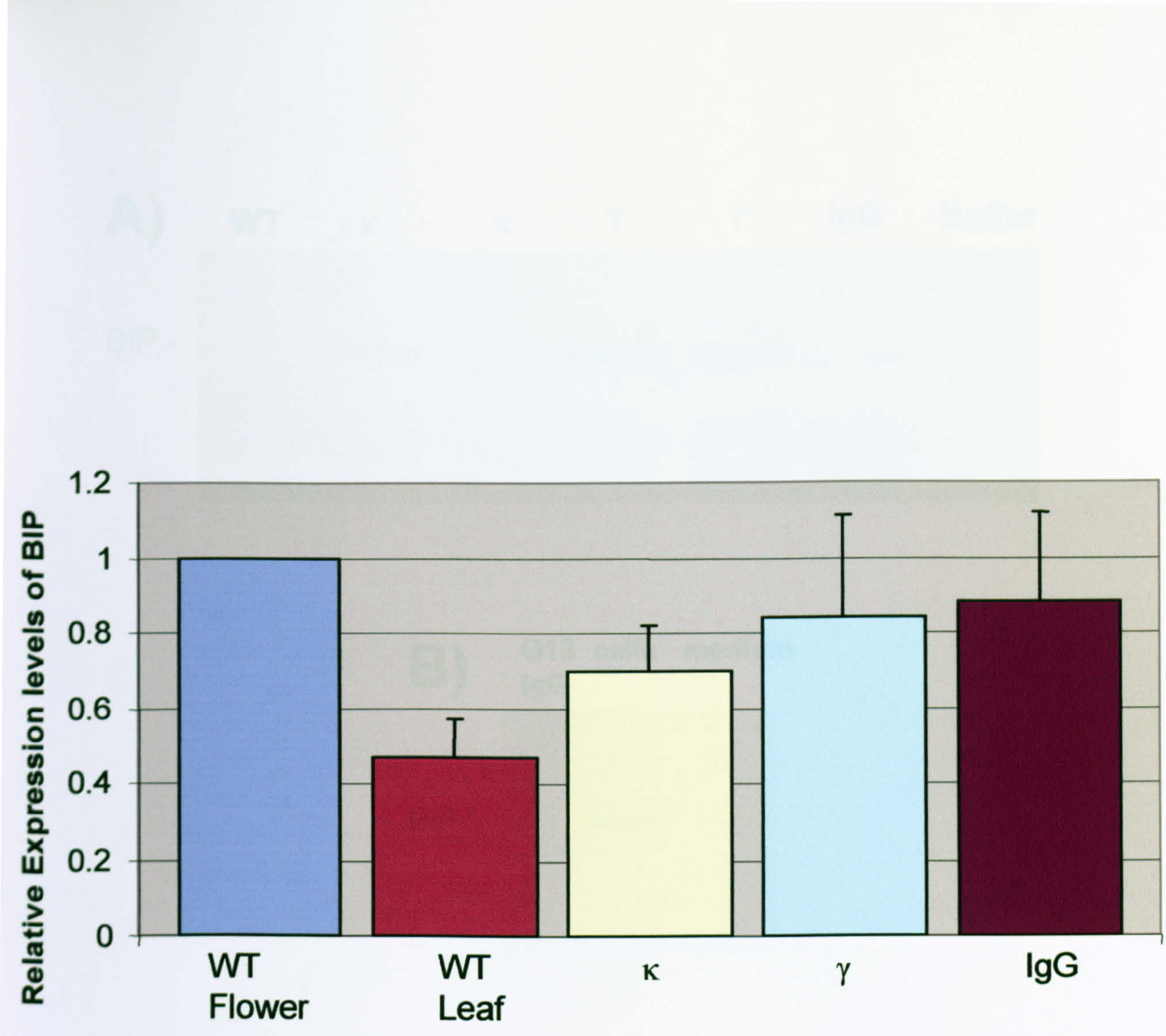


Figure 3.4: Relative levels of total BiP expression in leaf tissue from different plants. A standardised preparation of flower tissue was used as the 100 % level. Relative values were obtained by densitometric analysis of BiP-specific immunoblots. Data are shown as mean values for 6 plants + standard deviation.

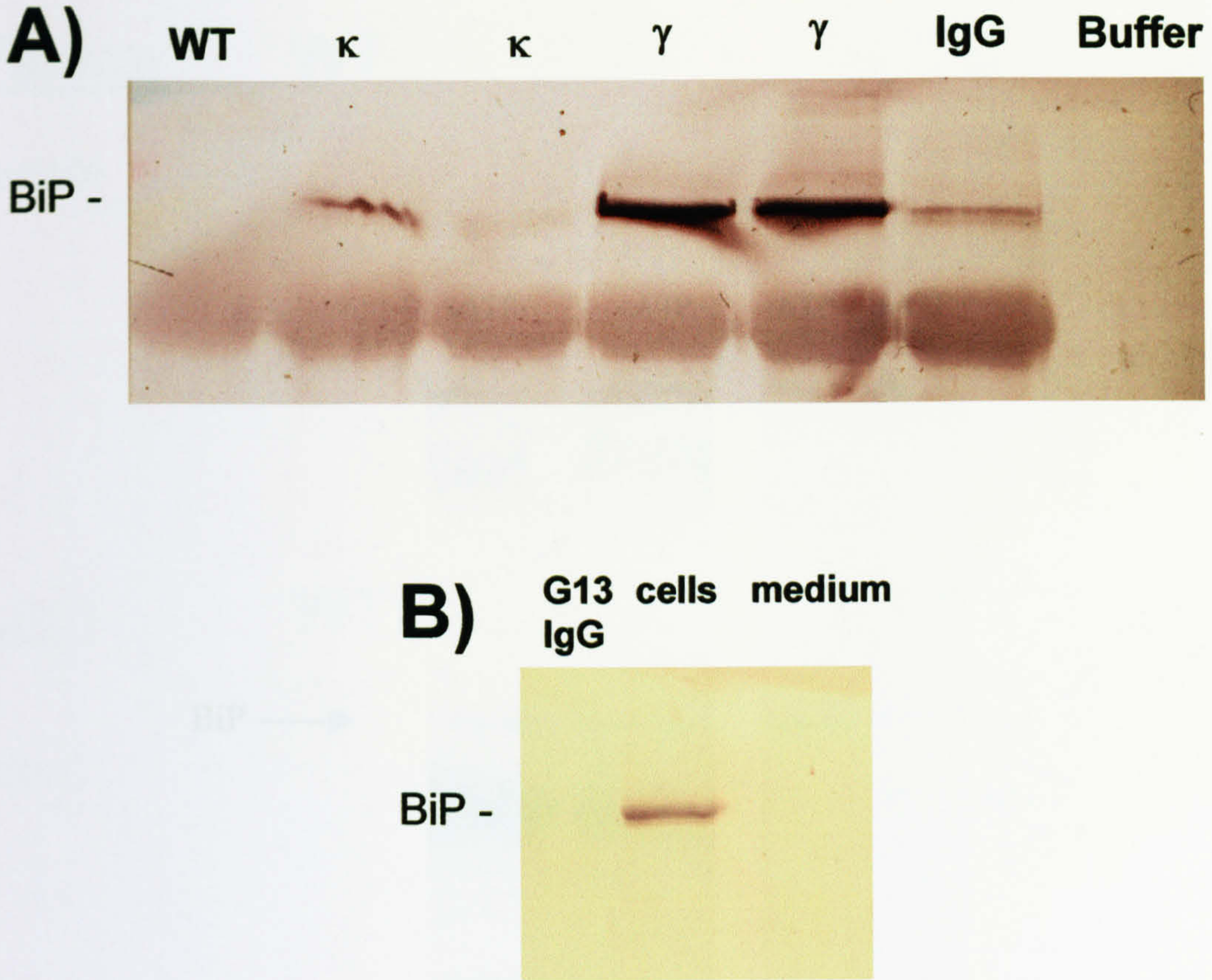


Figure 3.5: Detection of BiP associated with immunoglobulin chains by co-immunoprecipitation and western blotting.

A) Protoplasts were lysed and immunoprecipitation was with an anti-Mouse light and heavy chain antiserum. After purification with Protein A sepharose, samples were run on 10 % SDS-PAGE and blotted onto nitrocellulose. Detection was with a rabbit anti-tobacco BiP antiserum, followed by an alkaline phosphatase labeled anti-rabbit IgG antiserum. Samples were: wild type non-transformed protoplasts, κ chain transgenic plant protoplasts, g chain transgenic plant protoplasts, IgG transgenic plant protoplasts and protoplast lysis buffer. The expected position for BiP (M_r 75K) is indicated.

B) Immunoprecipitation was performed as above. Samples are Guy's 13 IgG hybridoma cell culture supernatant (G13 IgG), lysed protoplast preparation from IgG transgenic plant (cells) and the protoplast culture medium after overnight incubation with IgG transgenic protoplasts (medium). The expected position for BiP (M_r 75K) is indicated.

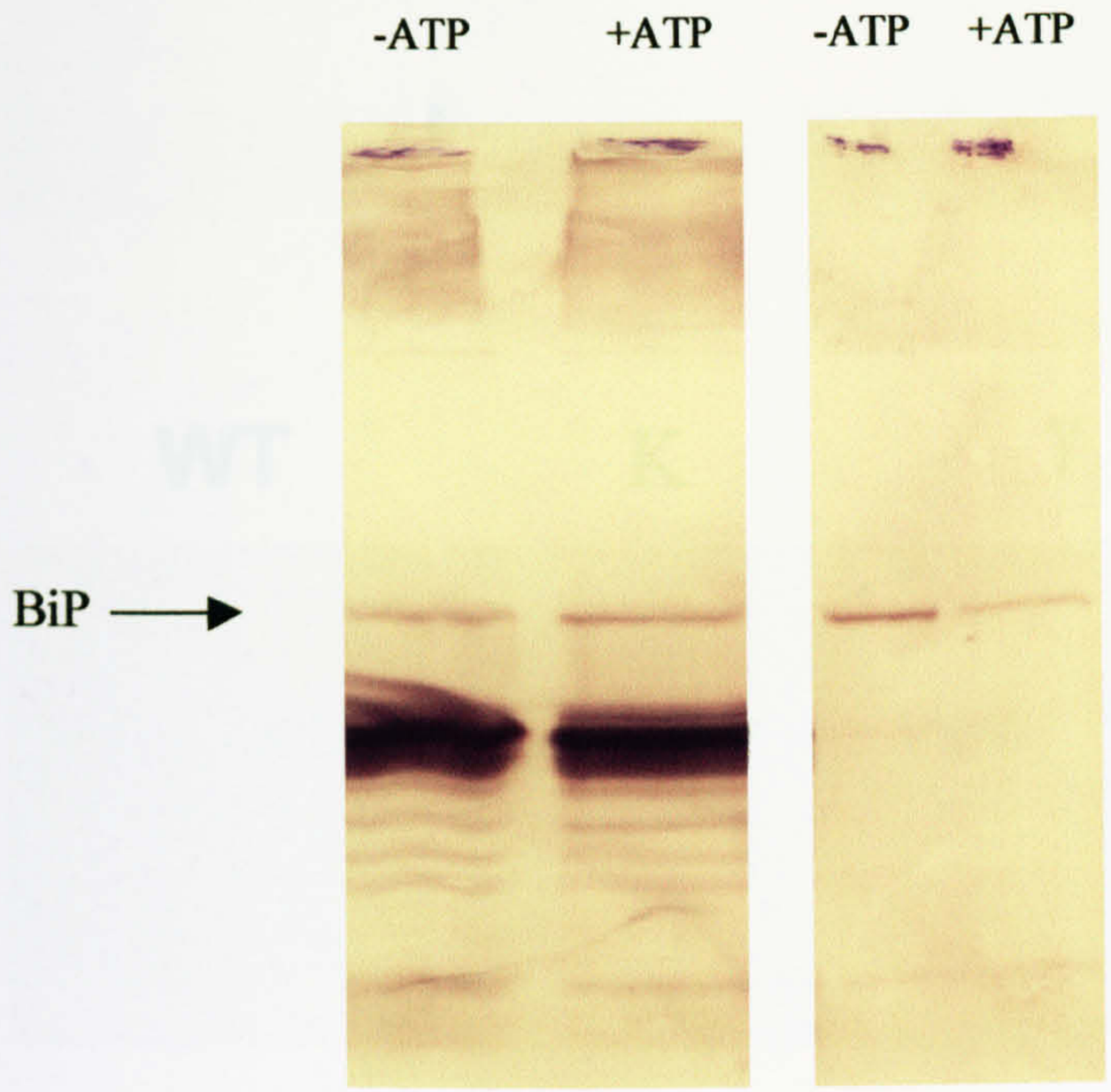


Figure 3.6: ATP release of BiP from recombinant immunoglobulin heavy chain in immunoprecipitates from gamma chain expressing protoplasts. Protoplasts were lysed and immunoselection was with either an anti-BiP or an anti Mouse IgG antiserum. After SDS-PAGE and transfer to nitrocellulose, detection was with a rabbit anti-BiP antiserum, followed by an alkaline phosphatase labeled anti-rabbit IgG antiserum. Results were consistent over several replicates.

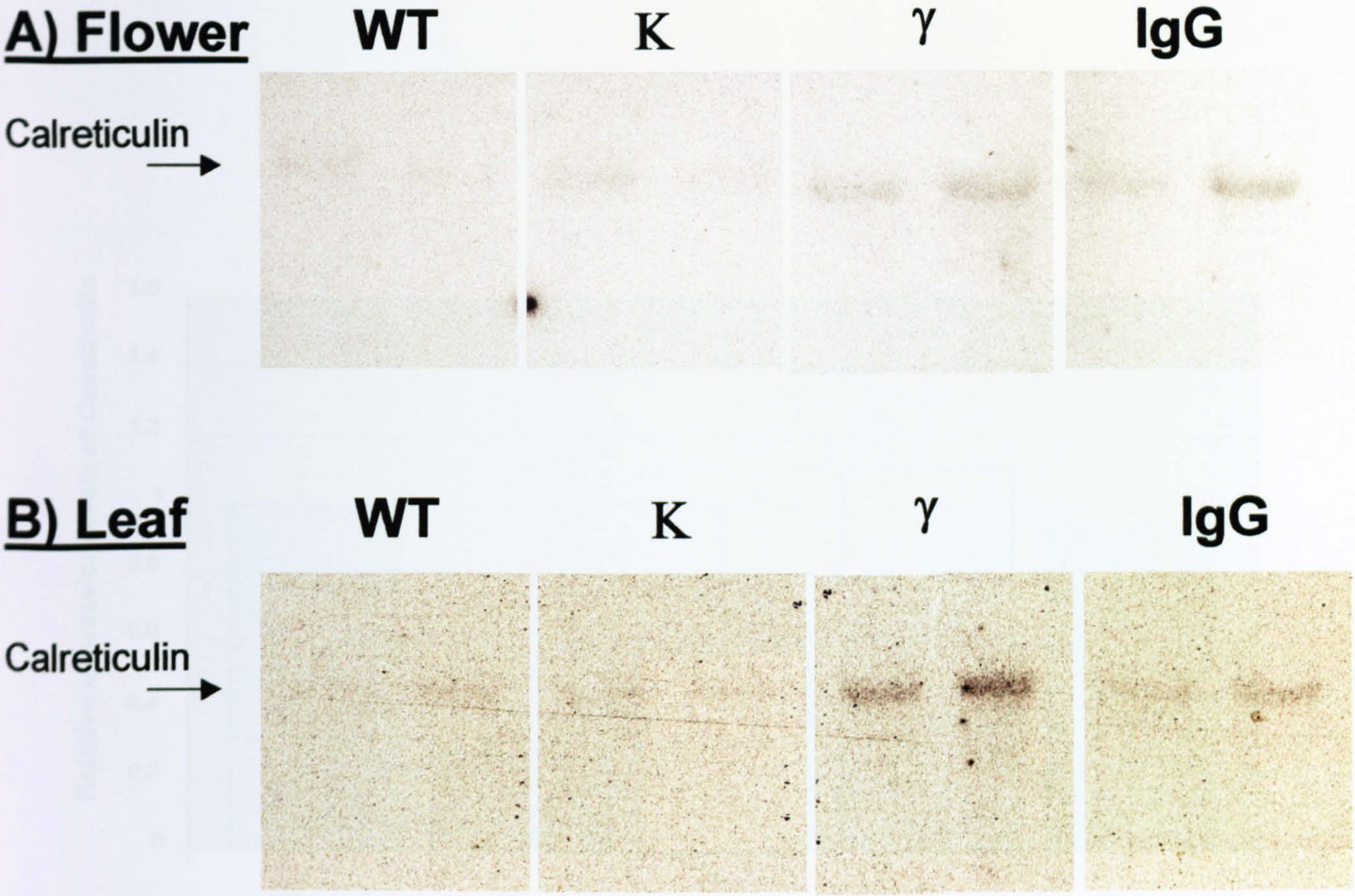


Figure 3.7: Detection of calreticulin transcript in plant RNA by Northern blotting. 15 μg total RNA from (A) flower tissue, (B) leaf tissue were run in each lane, blotted onto nitrocellulose and probed with a labeled calreticulin DNA probe. The expected position of calreticulin transcript is indicated. Each of the four panels in each set is from the same nitrocellulose blot and probed in an identical manner. The samples used were: wild type non-transformed plants, κ chain expressing plants, γ chain expressing plants and IgG expressing plants.

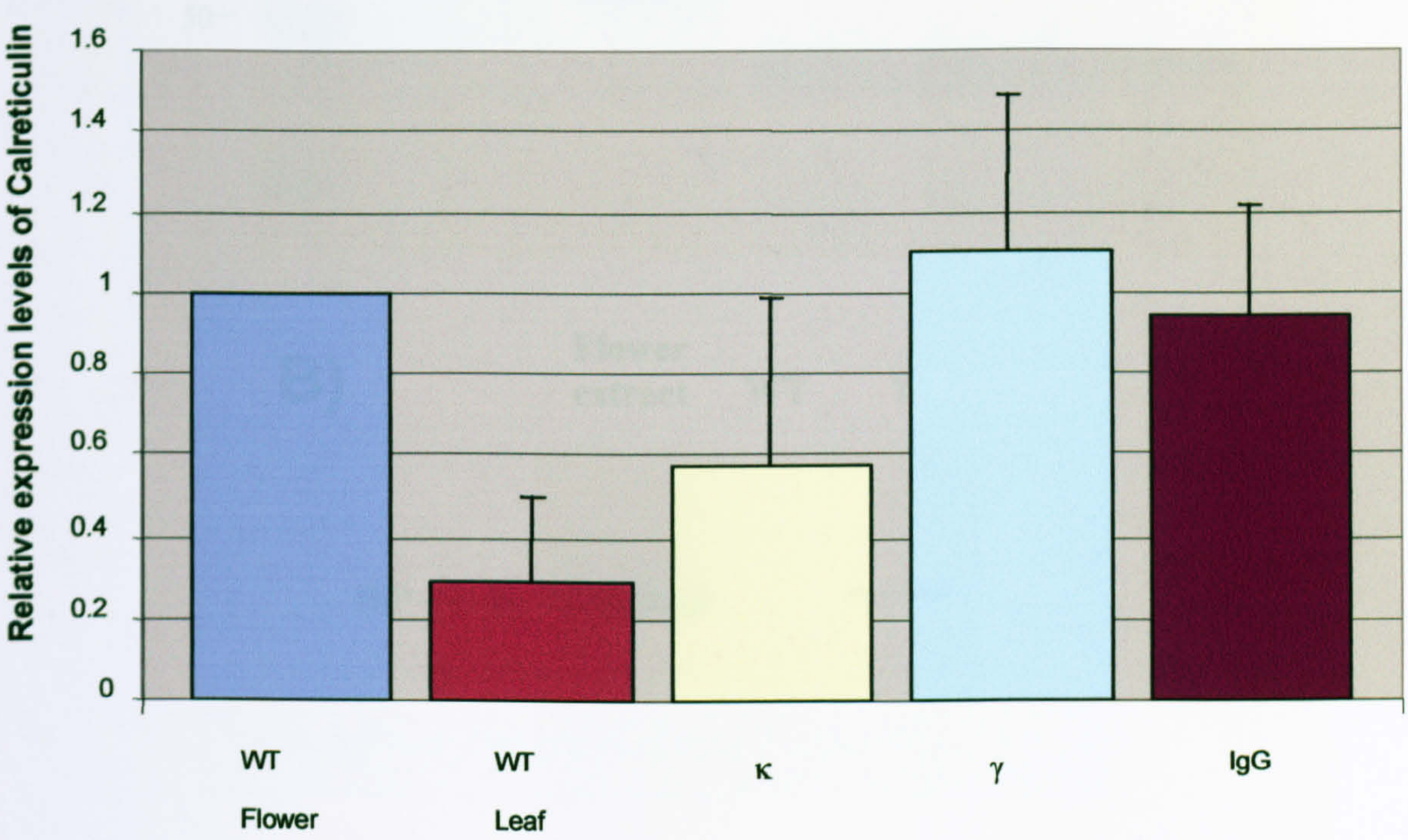


Figure 3.8: Relative levels of total calreticulin expression in leaf tissue from different plants. A standardised preparation of flower tissue was used as the 100 % level. Relative values were obtained by densitometric analysis of calreticulin-specific immunoblots. Data are shown as mean values for 6 plants + standard deviation.

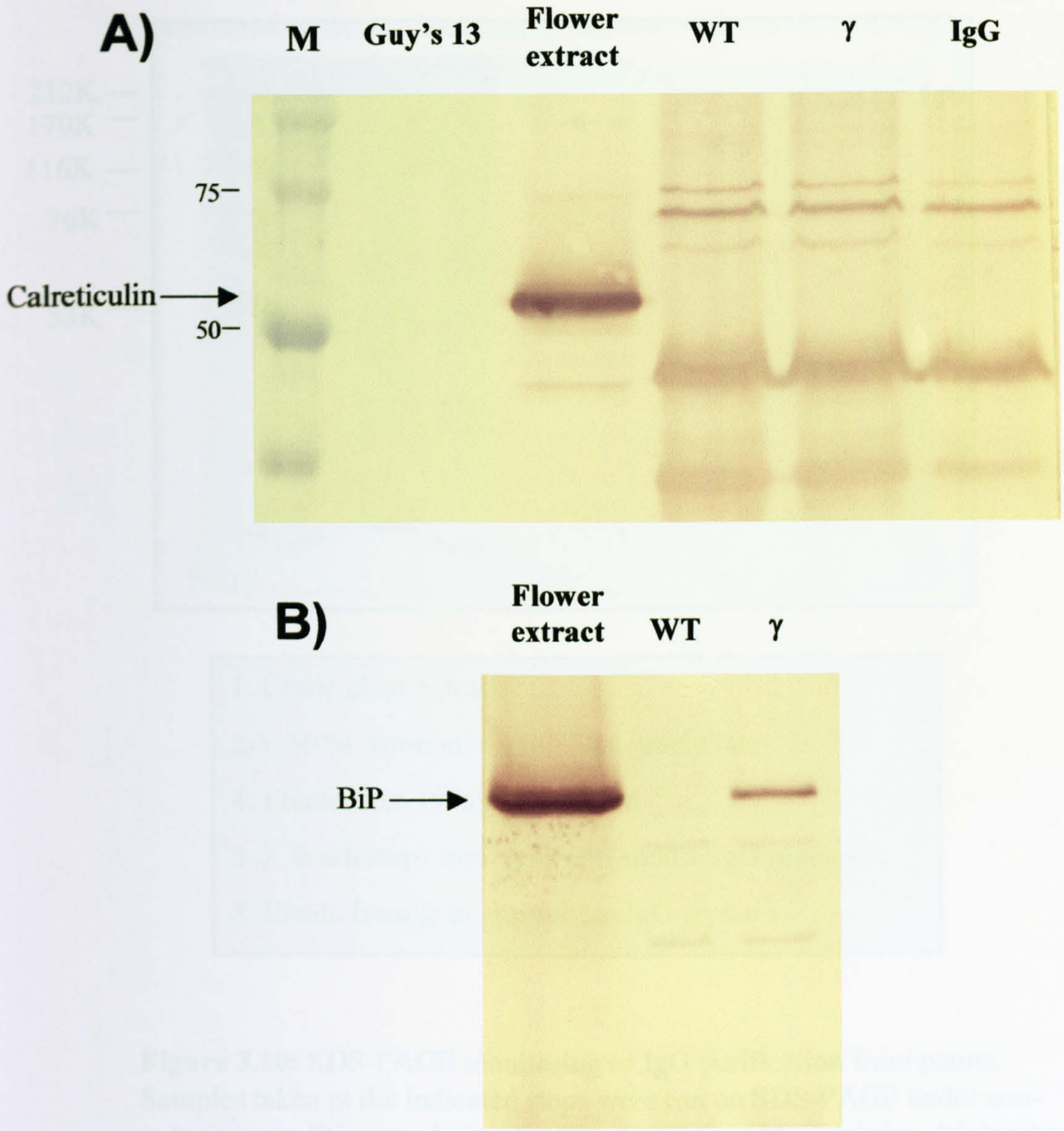
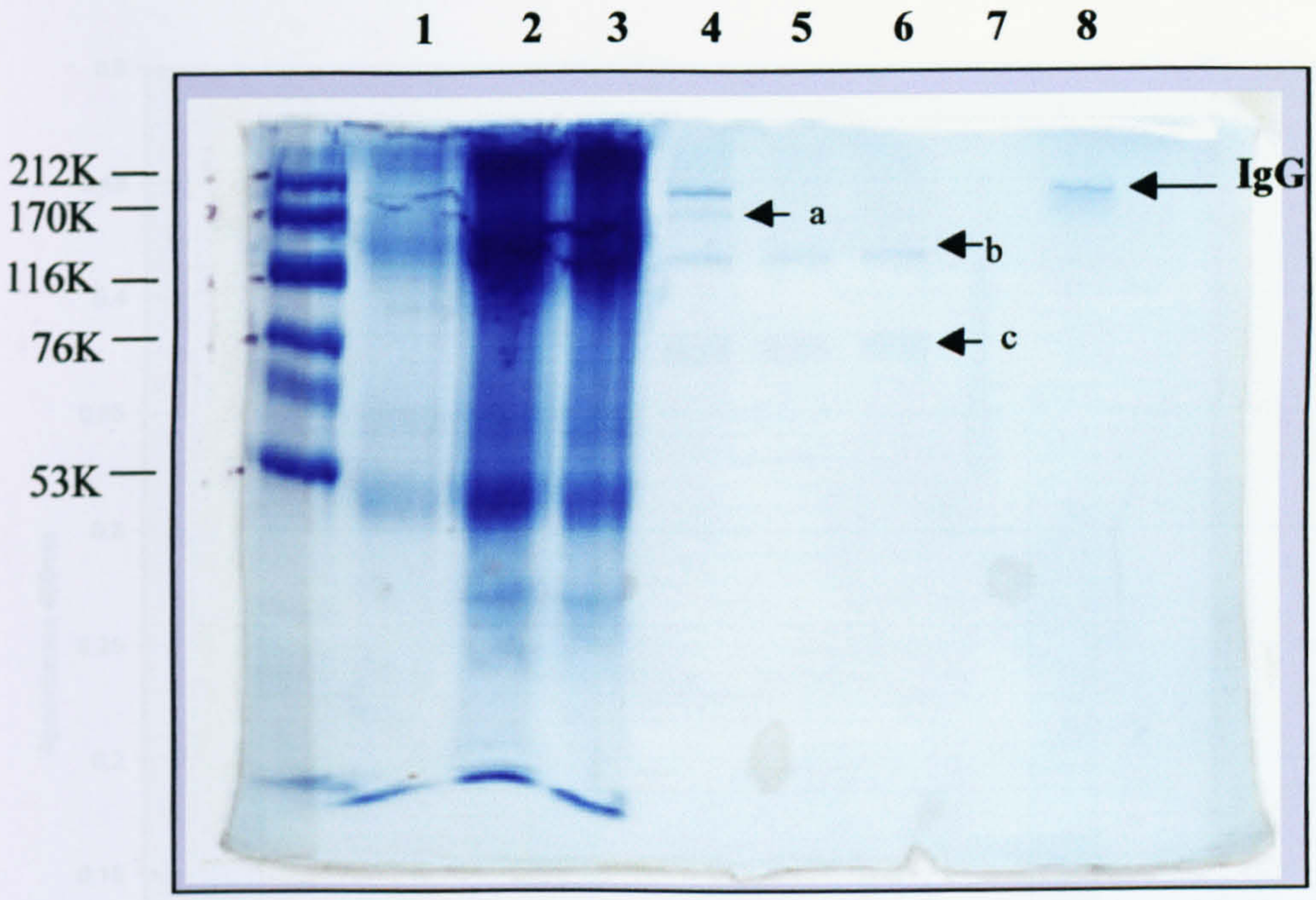


Figure 3.9: Western blot analysis of immunoprecipitates from plant protoplasts. The source material was transgenic plants. (A) The blot was probed with rabbit anti-calreticulin serum followed by alkaline phosphatase-conjugated anti-rabbit serum. A crude flower extract was included as a positive control for recognition by the antibody. (B) The blot was probed with anti-BiP serum followed by alkaline phosphatase-conjugated anti-rabbit serum. M, protein molecular size markers; G13 - Guy's 13 IgG hybridoma cell culture supernatant; WT - wild type non-transformed tobacco; γ - gamma chain transgenic tobacco; IgG - kappa and gamma chain transgenic tobacco.



1. Crude plant extract

2-3. 50 % Ammonium sulphate precipitate

4. Eluate from Protein G column

5-7. Wash steps from goat anti-mouse IgG sepharose

8. Eluate from goat anti-mouse IgG sepharose

Figure 3.10: SDS-PAGE monitoring of IgG purification from plants. Samples taken at the indicated steps were run on SDS-PAGE under non-reducing conditions and visualised by Coomassie blue staining. Molecular markers are indicated. The four bands labeled a,b,c and IgG are referred to in section 3.4.1.

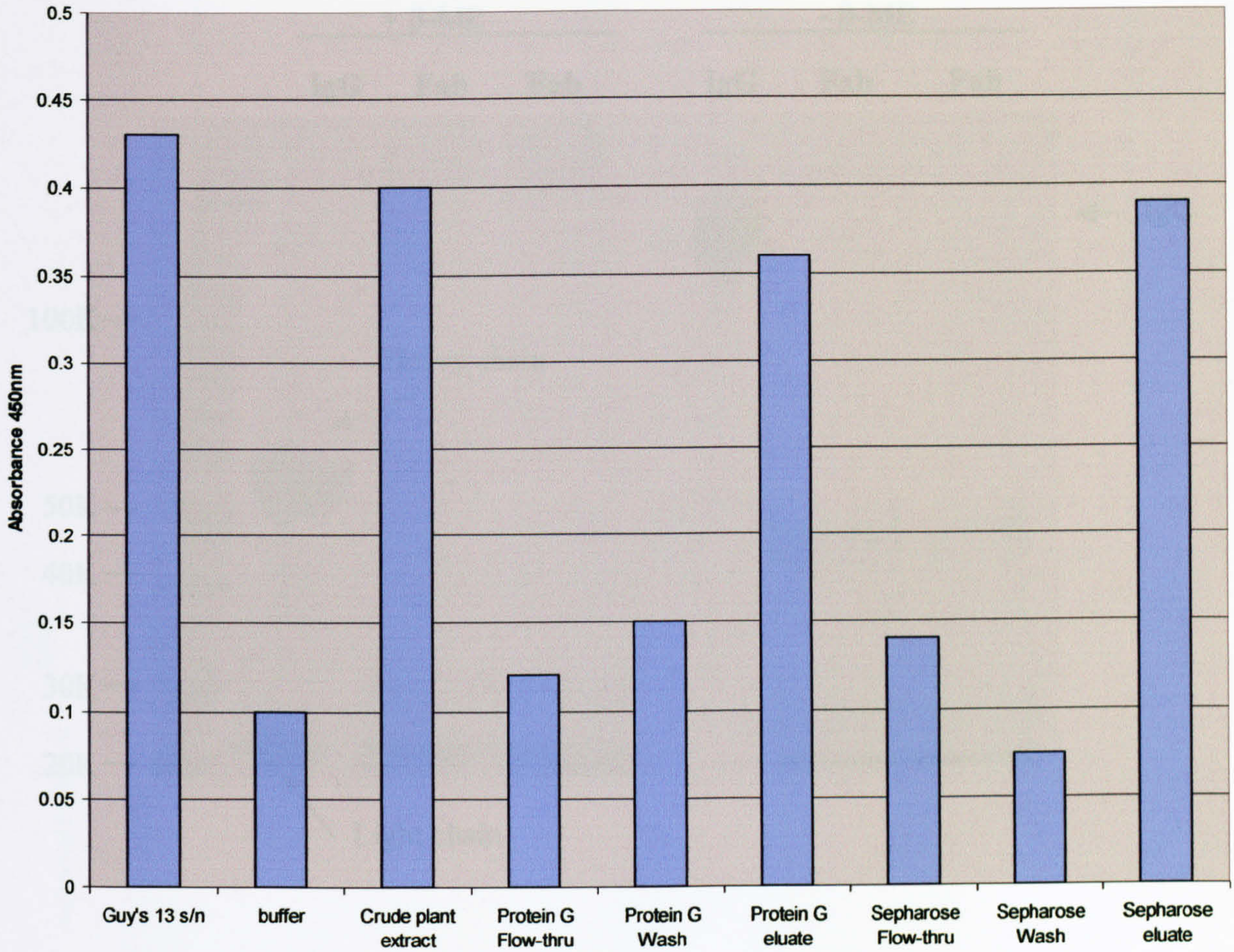


Figure 3.11: Antigen specific ELISA to monitor purification of antibody from plants. The samples indicated were incubated in ELISA wells pre-coated with streptococcal antigen I/II at 2 μg ml^{-1}. Detection of binding was with a horseradish peroxidase labeled anti-mouse IgG antiserum.

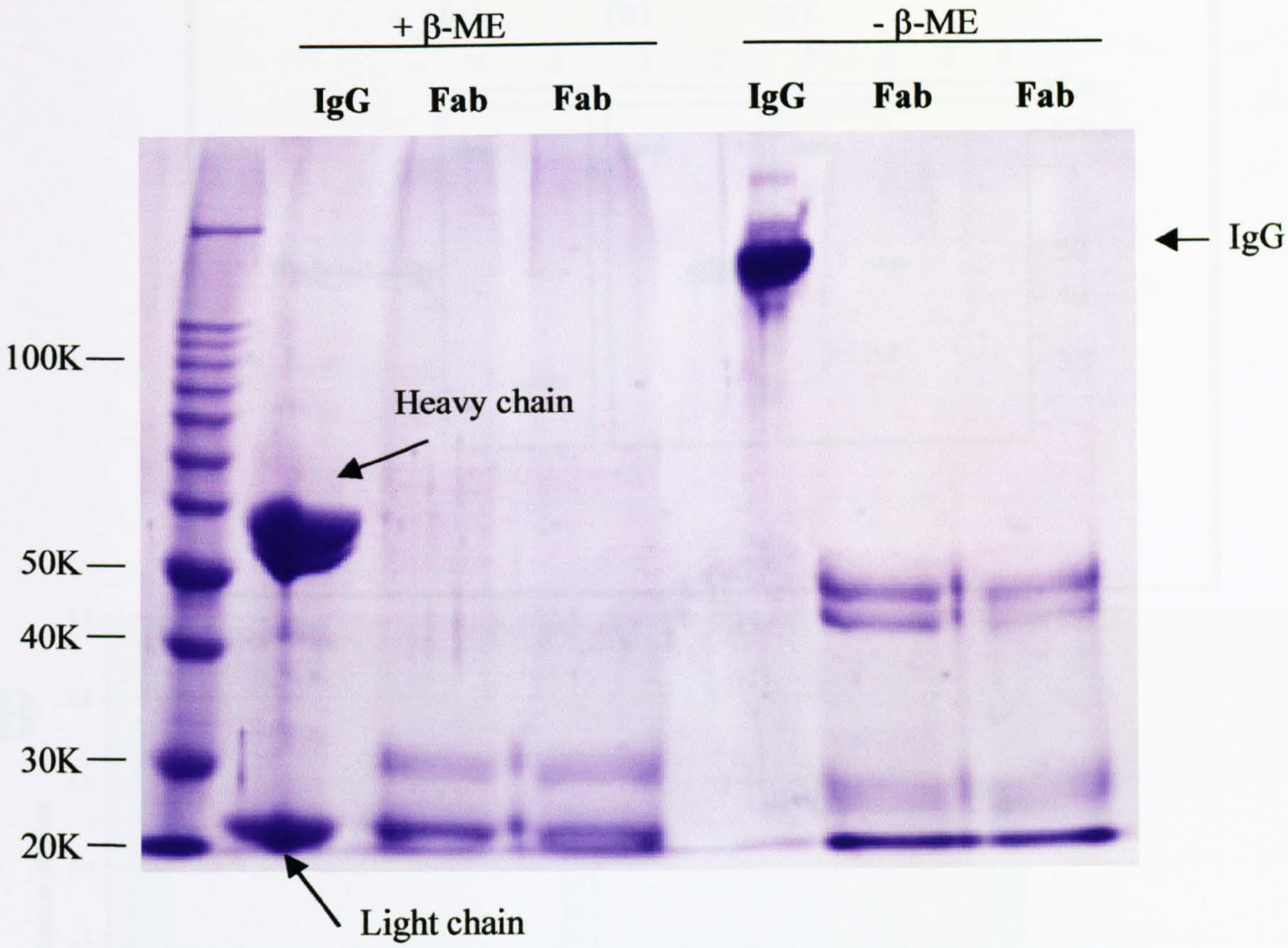
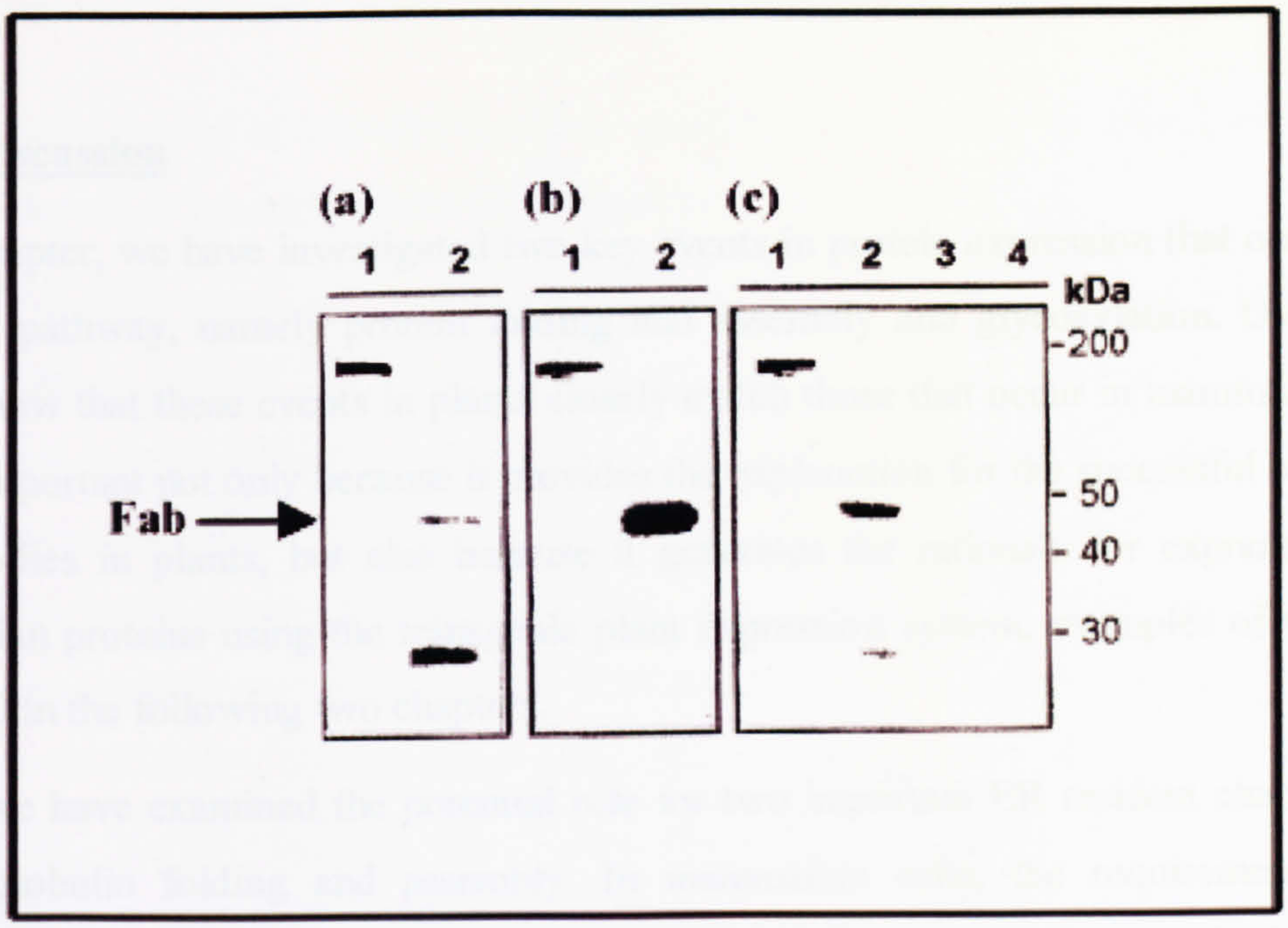


Figure 3.12: SDS-PAGE monitoring of papain digestions of plant derived IgG. Undigested (IgG) and digested (Fab) samples were run on SDS-PAGE under non-reducing (- β-ME) or reducing (+ β-ME) conditions and visualised by Coomassie blue staining. Molecular markers are indicated.

A



B

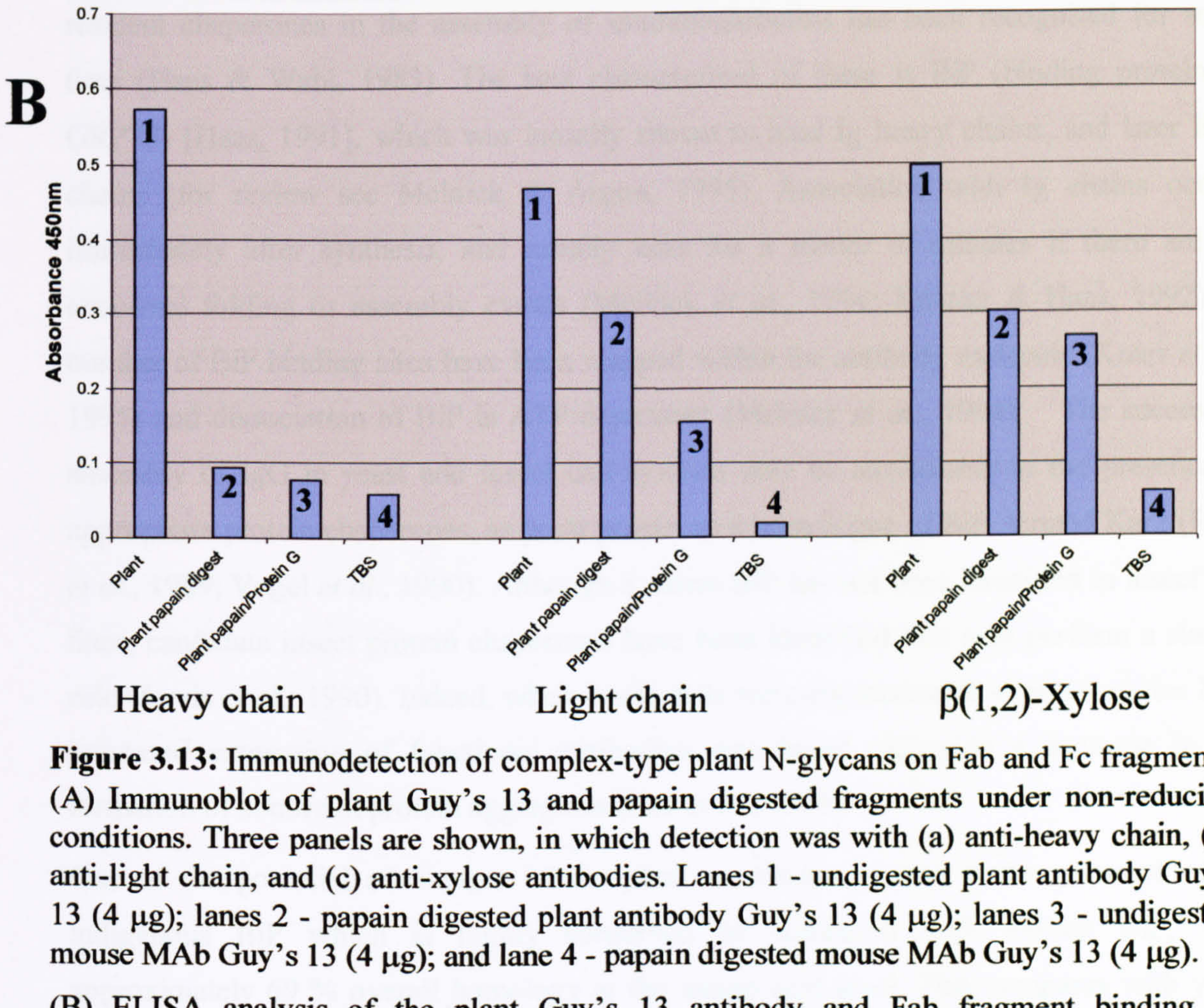


Figure 3.13: Immunodetection of complex-type plant N-glycans on Fab and Fc fragments. (A) Immunoblot of plant Guy's 13 and papain digested fragments under non-reducing conditions. Three panels are shown, in which detection was with (a) anti-heavy chain, (b) anti-light chain, and (c) anti-xylose antibodies. Lanes 1 - undigested plant antibody Guy's 13 (4 μg); lanes 2 - papain digested plant antibody Guy's 13 (4 μg); lanes 3 - undigested mouse MAb Guy's 13 (4 μg); and lane 4 - papain digested mouse MAb Guy's 13 (4 μg).

(B) ELISA analysis of the plant Guy's 13 antibody and Fab fragment binding to streptococcal antigen I/II. Detection was with either anti-heavy chain (Fc fragment)[1:16 dilution], anti-light chain (1:128 dilution) or anti-xylose (1:16 dilution) antibodies. Columns 1 - undigested plant antibody Guy's 13; Columns 2 - papain digested plant antibody Guy's 13; Columns 3 - papain digested MAb Guy's 13 absorbed by Protein G; and Columns 4 -TBS.

3.5 Discussion

In this chapter, we have investigated two key events in protein expression that occur in the secretory pathway, namely protein folding and assembly and glycosylation. Overall, the results show that these events in plants closely match those that occur in mammalian cells. This is important not only because it provides the explanation for the successful expression of antibodies in plants, but also because it generates the rationale for expressing other mammalian proteins using the transgenic plant expression system, examples of which are discussed in the following two chapters.

Firstly, we have examined the potential role for two important ER resident chaperones in immunoglobulin folding and assembly. In mammalian cells, the requirement for ER resident chaperones in the assembly of immunoglobulins has been recognised for some time (Haas & Wabl, 1983). The best characterised of these is BiP (binding protein or GRP78) [Haas, 1991], which was initially shown to bind Ig heavy chains, and later light chains (for review see Melnick & Argon, 1995). Association with Ig chains occurs immediately after synthesis, and usually lasts for a matter of minutes if there are no abnormal folding or assembly events (Melnick *et al.*, 1994; Knittler & Haas, 1992). A number of BiP binding sites have been mapped within the antibody molecule (Knarr *et al.*, 1995) and dissociation of BiP is ATP-dependent (Melnick *et al.*, 1994). The successful assembly of IgG in yeast and insect cell systems may be attributable to the presence of appropriate protein chaperones, as yeast possesses a homologue of BiP, termed Kar2 (Rose *et al.*, 1989; Vogel *et al.*, 1990). Although a native BiP has not been identified in insect cell lines, candidate insect protein chaperones have been identified that may perform a similar role (Jarvis *et al.*, 1990). Indeed, when insect cells were engineered to express murine BiP, increased expression of functional antibodies was found alongside a decrease in the formation of abnormal protein aggregates (Hsu *et al.*, 1994).

One of the potential advantages of the plant expression system is the presence of an indigenous BiP which is highly conserved as compared with murine BiP, with approximately 69 % overall homology at the amino acid level. This compares with yeast BiP which has 64 % overall homology with murine BiP. In tobacco, BiP mRNA expression is highest in tissues containing rapidly dividing cells or those that are involved in secretion (Denecke *et al.*, 1991), whereas in maize, BiP is expressed most abundantly in endosperm

development (Fontes *et al.*, 1991). Also, plant BiP expression is associated with the accumulation of abnormal proteins (Pedrazzini *et al.*, 1994).

In plants it was previously demonstrated that efficient assembly and expression of immunoglobulins could only be achieved by using a leader sequence to target the recombinant immunoglobulin proteins to the ER and the secretory pathway (Hiatt *et al.*, 1989). This might be due to enhanced translation of recombinant proteins or to increased stability of the proteins resulting from their sub-cellular localisation. In this chapter, evidence has been put forward that demonstrates the association of the plant BiP homologue with folding and assembly of Ig light and heavy chains and we propose that the involvement of ER-resident chaperones promotes processing and expression of immunoglobulin molecules in plants.

Previous analysis of the constitutive expression patterns of BiP mRNA has suggested that expression is low in tobacco leaves (Denecke *et al.*, 1991). For this reason, in these investigations we have used leaf tissues initially, so that any elevation in BiP would be more readily detected over the low background of constitutive expression. BiP mRNA was differentially expressed in four plant lines that expressed no transgenic product, Ig light or heavy chain, or assembled IgG. The highest BiP expression was found in plants expressing heavy chain only, BiP expression was relatively lower in plants that express both light and heavy chains, but still elevated as compared with non-transgenic plants. The results are consistent with the putative role of BiP in binding to and retaining unassembled sub-unit proteins (Haas & Wabl, 1983; Knittler *et al.*, 1995) and in folding and assembly of immunoglobulin chains in cells that are highly active in terms of recombinant protein production and secretion. The relative decrease in BiP mRNA in IgG expressing plants as compared with heavy chain expressing plants might be attributable to the successful assembly of heavy chains into immunoglobulin. This reduces the levels of non-assembled heavy chain, and allows the release and recycling of BiP.

Immunoblotting experiments also appeared to demonstrate a trend in the expression profiles of the total amounts of BiP and calreticulin protein present in transgenic leaf extracts. Lower levels of BiP and calreticulin protein were detected in wild type plants, and plants expressing κ light chains. Higher levels of BiP and calreticulin protein were detected in transgenic plants expressing γ heavy chains, and assembled IgG. These differences might be explained simply by differences in expression levels of unassembled light and heavy

chains, and assembled IgG. In the original experiments which demonstrated the production of antibodies in transgenic plants (Hiatt *et al.,* 1989), the yield of antibody chain was increased in plants expressing both γ and κ chains, indicating that assembly of the γ-κ complex might enhance stability. Our ELISA results support this finding, and also confirm that the level of κ chain expression is much lower than that of γ chains. However, the expression levels of assembled IgG were approximately 6-fold greater than that of γ heavy chains alone, but this was not reflected in the levels of total BiP or calreticulin detected. This indicates that chaperone levels in transgenic plants expressing immunoglobulin chains are regulated by other factors.

When we examined co-immunoprecipitation between BiP or calreticulin and recombinant immunoglobulin chains, we found convincing evidence for an interaction between BiP and light and heavy chains, but not for calreticulin. BiP protein co-precipitated with immunoglobulin chains extracted from protoplast intracellular fluid, but not from secreted Ig chains. This indicates that the BiP-Ig chain interaction in plants is not artefactual and is consistent with the model of BiP binding transiently in the ER to Ig chains during protein folding and processing. The results matched those found by Northern blot, in that most BiP protein was immunoprecipitated in plants expressing the Ig heavy chain only. Less was detected from plants expressing both light and heavy chains, even though more Ig heavy chain protein is consistently recoverable from IgG expressing plants. Again this is consistent with the model for BiP in assisting protein folding and increasing throughput of the ER.

In mammals, BiP associates with both Ig light and heavy chains (Knittler & Haas, 1992; Haas & Wabl, 1983), and by immunoprecipitation, similar results were found in plants. The relatively weak signal observed may have been because the immunoprecipitation protocol was optimal for heavy chains and not light chains. However in view of the finding that BiP mRNA expression was not elevated in light chain-expressing plants, an alternative explanation is that the levels of light chain accumulation are generally very low in single transgenic plants. Previous estimates of the accumulation levels of light chain have generally been 20-fold lower as compared with plants expressing light and heavy chains together (Hiatt *et al.,* 1989).

We have also investigated whether calreticulin might be involved in the chaperoning of immunoglobulins in plants and found no evidence. Although we cannot exclude the

possibility that an extremely rapid interaction occurs between calreticulin and immunoglobulin chains, our findings appear to be consistent with mammalian expression of immunoglobulins (Melnick & Argon, 1995), and demonstrates a specific and appropriate interaction between immunoglobulins and chaperones in plants. There is increasing evidence for separate chaperone pathways involving either BiP or calreticulin/calnexin (Molinari & Helenius, 2000) and it remains to be determined if the plant calreticulin pathway is equally functional for mammalian proteins.

We have been able to confirm and extend these studies with BiP through a collaboration with Dr. Lorenzo Frigerio at the University of Warwick (Nuttall *et al.*, 2002). Using the transient expression system in protoplasts, the co-immunoprecipitation of BiP with immunoglobulin heavy chains in an ATP dependent manner was confirmed. An important finding in this study, was further confirmation of the functional nature of the interaction between BiP and immunoglobulin heavy chain. To test the hypothesis that the co-expression of the companion κ chain should compete with BiP for association with the IgG heavy chain, tobacco protoplasts were co-transfected with a fixed amount of DNA encoding heavy chain and with increasing amounts of light chain-encoding DNA. The results showed that when heavy chain (γ) expression is constant, an increase in light chain (κ) expression is paralleled by a decrease in the amount of BiP that is co-selected from the cell homogenates. When the same samples were run on non-reducing SDS-PAGE, it was demonstrated that co-transfection of the light chain resulted in the assembly of the IgG heterotetramer in a dose dependent manner. Therefore, the presence of the light chain triggers assembly of the IgG tetramers and causes BiP to be partially released from the heavy chains.

Surprisingly, no interaction was observed between BiP and light chain when light chain was transiently expressed in protoplasts alone. The difference in results between transient expression and those found in our transgenic plant protoplasts may be due to a difference in expression levels of immunoglobulin light chain between the two systems. Alternatively, it may reflect a more rapid turnover of transiently expressed light chains, which would lead to its interaction with the pool of unlabelled BiP within the time frame of our observation. Preliminary data suggest that in plants expressing only Ig light chains, these light chains are normally secreted (Frigerio, unpublished). In contrast when expressed alone, heavy chain accumulates intracellularly and co-localises in the ER with BiP. When assembled, the

immunoglobulin complex is secreted (Frigerio *et al.*, 2000). Thus BiP may be involved in the chaperoning of light chains in plants, but as the protein is efficiently secreted, the detectable signal in the BiP co-immunoprecipitation assay may be too low.

The association of BiP with folding and assembly of recombinant immunoglobulin chains in plants is significant. Protein translocation and folding in the ER can be one of the rate limiting steps in protein secretion, and the presence of protein chaperones is important for high efficiency turnover, leading to high levels of production. It has been reported that the overexpression of BiP (and PDI) in yeast cells greatly improves the efficiency of folding and secretion of single chain antibody fragments (Shusta *et al.*, 1998). Likewise, when BiP is overexpressed in transgenic plants, it is able to alleviate ER stress induced by tunicamycin (Leborgne-Castel *et al.*, 1999). It will be very interesting to test the effects of BiP overexpression in plants expressing our model immunoglobulins.

The passage of proteins through the secretory pathway in plants is a complicated process (Vitale, 2001) and it is clear that BiP is not the only chaperone involved. In mammals, a few other chaperones that are involved with immunoglobulins have been identified so far (Melnick & Argon, 1995; Melnick *et al.*, 1994). Of these, plant homologues to GRP 94, and protein disulphide isomerase (Shorrosh & Dixon, 1991) have been identified, and it will be important to establish their role in Ig assembly.

The results from our experiments suggest that in transgenic plants expressing recombinant immunoglobulins, BiP and calreticulin might be coordinately regulated. However a chaperone function for calreticulin in immunoglobulin assembly has not been identified. BiP and calreticulin represent a large proportion of the steady state content of the ER lumen and can be considered as a measure for ER abundance (Denecke *et al.*, 1995). ER-resident chaperones, such as BiP, are thought to be regulated through a feedback mechanism known as the malfolded protein response. BiP has been found in association with calreticulin, both in the presence and absence of ER stress (Crofts *et al.*, 1998). The BiP-calreticulin complex was stable *in vivo*, and BiP appears to be the binding protein and calreticulin the ligand. Although it was suggested that the BiP-calreticulin complex could provide a buffer for the concentration of free BiP, the precise function of the complex is not clear.

In the second part of this investigation of immunoglobulin processing through the plant secretory pathway, we have investigated the presence of glycans at N-glycosylation sites

that are known to be utilised in the murine system. Many mammalian proteins have been successfully produced in plants, demonstrating the fidelity with which plants can express transgenes and fold and assemble the protein product. Glycosylation is another important area in which a level of fidelity is required, in order to maintain function and biological activity. Thus the potential use of plants as expression systems for the production of therapeutic recombinant proteins is often dependent on the ability of plants to produce and glycosylate mammalian glycoproteins.

In order to perform these studies, it was necessary first to establish a methodology for purification of IgG from plants, to avoid contamination with other plant glycoproteins. There are several problems inherent and unique to purifying rAbs from plants. Some of these difficulties are caused by the presence of polyphenolic compounds, such as tannins. Furthermore, once plant tissue is damaged in order to extract recombinant proteins, a number of potentially toxic vacuolar contents are also released. These include secondary metabolites, such as nicotine in tobacco, proteases, polysaccharides, lipids, and DNA, all of which may reduce protein yield. These chemicals may additionally have a toxic effect or cause sensitisation of humans to the plant antigens. Effective methods for the removal of phenolic compounds from aqueous plant extracts are being developed, for example polyamide minicolumns that remove more than 99 % polyphenolic compounds from 5 mg of extract (Collins *et al.*, 1998).

A purification protocol for a TMV-specific full-length rAb from a transgenic tobacco plant cell suspension culture has been described (Fischer *et al.*, 1999a). The antibody was derived from a stably transformed transgenic plant, and although targeted for secretion by a murine leader sequence, was retained by the cell wall in suspension culture. The primary plant cell wall was partially digested by enzymatic treatment, resulting in a total release of the rAb into the extraction buffer. Purification of the IgG was achieved by cross-flow filtration, followed by protein A affinity chromatography, which lead to a 100-fold concentration of the rAb and efficient removal of contaminants (Fischer *et al.*, 1999a). Gel filtration was used as a final purification step, and more than 80 % of the expressed full-length IgG from plant cell extracts was recovered.

For the purposes of our studies, we were able to purify approximately 1 mg IgG from plants, using standard methodologies of protein concentration by ammonium sulphate precipitation, followed by affinity chromatography. We were able to achieve purification to

two bands on Coomassie blue stained SDS polyacrylamide gels.

It was already known that MAb Guy's 13 contains two potential N-glycosylation sites on the heavy chain, located on the Fc and Fab fragments, both of which are glycosylated in the murine version of the antibody. Using a functional antigen-specific ELISA and immunoblotting we showed that plant complex N-glycans are also located on both the Fc and Fab fragments of the plant Guy's 13 antibody. This plant specific glycosylation was sufficient to generate soluble and biologically active IgG.

Using the same purification protocol, we have scaled-up purification to 10 mg IgG, which allowed a detailed structural analysis of the N-linked glycans associated with Guy's 13, and a comparison between murine and plant versions of the same antibody by our collaborators at the University of Rouen (Cabanes-Macheteau *et al.*, 1999). We found that whereas the mouse Guy's 13 contained only 4 different complex-type glycans, the plant Guy's 13 was more heterogeneous in glycosylation. Eight different oligosaccharides were identified on the plant antibody, representing an array of structurally related oligosaccharides from high-mannose-type N glycans (identical to mammalian high-mannose glycans), to complex structures containing the β(1,2)-xylose and α(1,3)-fucose residues that are characteristic of plant glycoproteins. N-acetylglucosamine residues were the major oligosaccharides N-linked to the recombinant plant Guy's 13 antibody, a finding that is consistent with secretion of the molecule from plant cells (Lerouge *et al.*, 1998). However the large structural heterogeneity of the recombinant plant glycans could also be the consequence of the extraction of the antibody from other compartments of the plant secretory pathway. Previously it was shown that storage of the recombinant plant antibody in the endoplasmic reticulum or in the vacuole results in N-glycans having unprocessed or truncated structures (Lerouge *et al.*, 1996).

The differences observed in the glycosylation pattern of the plant version of Guy's 13 MAb compared with the murine version have no effect on antigen binding or specificity. However, it was suggested that these differences could represent an important limitation to the use of plant recombinant mammalian glycoproteins if the plant glycans are immunogenic (Bardor *et al.*, 2003). The potential immunogenicity of a plant derived antibody has indeed been investigated in a clinical trial using human subjects, to be described more fully in the Discussion section of Chapter 5. A purified form of Guy's 13 MAb expressed in plants was topically applied to the oral cavity of human volunteers on

six occasions. No evidence of an immune response to the plant recombinant glycoprotein was detected (Ma *et al.*, 1998).

In order to investigate the potential systemic immunogenicity of plant glycans, Dr. Daniel Chargelegue in our laboratory made use of the plant derived Guy's 13 MAb expressed in this study, to perform a parenteral challenge in mice (Chargelegue *et al.*, 2000). The same strain of mice (BALB/c) was used in this study as had been used to generate the original Guy's 13 MAb, thus allowing analysis of the immunogenicity of a self-protein displaying foreign plant glycans. Four groups of six mice were immunised subcutaneously with 30 μg mouse Guy's 13 MAb, the same antibody produced in transgenic tobacco plants, affinity purified horseradish peroxidase (HRP) - a standard plant glycoprotein - or PBS. HRP contains both of the predominant immunogenic plant carbohydrates, α(1,3)-fucose and β(1,2)-xylose, and was used as a positive control for plant glycoprotein. Immunisations were performed in the presence of 50 % alum as an adjuvant, to increase the chances of generating an immune response. Antibody responses were analysed by ELISA, and BIAcore studies, with pre-immune sera used as negative controls.

The mice immunised with HRP raised high IgG titres to HRP, although these antibodies did not cross-react with recombinant plant Guy's 13 antibody. None of the mice immunised with mouse Guy's 13 raised detectable antibody responses to mouse Guy's 13, plant Guy's 13 or HRP. In the group immunised with recombinant plant Guy's 13, two out of six mice had low reactivity to plant recombinant Guy's 13 as detected in ELISA. When these two positive sera were diluted 1/5 and tested for their reactivity to plant glycans, neither inhibited the binding of the anti-HRP rabbit immune-sera to recombinant plant Guy's 13. As these sera did not react to the mouse Guy's 13 MAb either, it was suggested that these results represented reactivity to small residual amounts of tobacco protein present at the end of the purification of the plant recombinant antibody. For future clinical studies, it will be important to make sure that these contaminants are removed before immunisation, perhaps by a final ion-exchange step or by gel filtration HPLC chromatography.

Although no haematological or serological adverse effects were detected following the human clinical trial of the mouse Guy's 13 MAb expressed in plants (Ma *et al.*, 1998), it is still conceivable that sensitisation to the plant complex-type N-glycans on the plant Guy's 13 antibody might take place. In the future it might be advantageous to generate a recombinant plant antibody with a carbohydrate profile structurally more consistent to one

derived from mammalian cells. This could involve removing the complex glycans altogether by altering the heavy-chain sequence and removing the site for N-linked glycosylation. Alternatively, the use of mutant plants lacking enzymes intrinsic to the glycosylation pathway might be a solution to this problem (Von Schaewen *et al.*, 1993). Progress has also been made towards "humanising" the glycosylation pathway in plants (Bakker *et al.*, 2001).

CHAPTER 4:

RESULTS II

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CHAPTER 4: RESULTS II

Targeting and retention of recombinant proteins to plant cell membranes

We have previously shown that the default pathway for IgG molecules in plants involves secretion out of the cell with extracellular accumulation, probably in the apoplastic space (Chapter 3 and Frigerio *et al.*, 2000). This is identical to the fate of immunoglobulin molecules produced in mammalian plasma cells. However, in mammalian cells (B cells and plasma cells), another form of antibody is also found, as a surface antigen receptor. This membrane associated form differs from the secreted form, only by having slightly larger heavy chains than those of secreted antibody, due to the presence of an extra 71 amino acid residues at the carboxyl terminus of each heavy chain. The extra amino acid residues are arranged in 3 domains that are highly conserved within the murine Cγ1, Cγ2a and Cγ2b as well as the Cμ genes - a 17 residue acidic extracellular hinge region, a 26 residue hydrophobic intra-membrane portion, and a 28 residue hydrophilic intracellular portion (Yamawaki-Kataoka *et al.*, 1982).

The aims of this part of the study were to:

1) determine if a murine Ig heavy chain could be targeted to the plant cell membrane using a murine Ig membrane retention sequence.

2) to examine if a functional antibody could be assembled and retained at the plant cell membrane, and to investigate the effect of this retention sequence on the expression of Ig heavy chain and assembled IgG in transgenic tobacco plants.

3) to extend the investigation of recombinant protein targeting to the plant plasma membrane, using a non-immunoglobulin mammalian membrane protein – CCR-5 a 7-transmembrane protein, in order to explore further the capacity for plants to accommodate heterologous recombinant membrane proteins.

The transgenic plants used in this part of the study were those expressing;

1) Guy's 13 MAb light chain (κ)

2) Guy's 13 MAb heavy chain (γ1), amino acids 1 – 457

3) Guy's 13 MAb membrane heavy chain (mγ1), amino acids 1 – 528, including the 71 amino acid membrane retention sequence

The sequence of the murine membrane retention polypeptide is shown in Figure 4.1 along with a diagrammatic representation of the three domains – a short hinge region, the transmembrane sequence and an intracellular tail.

The agrobacterium mediated transformation and regeneration of these plants were as described (Ma *et al.*, 1994; Vine *et al.*, 2001). As before, antibody expressing plants were produced by crossing the light chain plant with either the heavy (γ1) chain plant to generate IgG plants, or with the membrane heavy (mγ1) chain plant to generate mIgG plants. In all experiments, non-transformed (WT) plants were included as a negative control.

4.1 Detection of recombinant immunoglobulin chains by ELISA

Crude plant extracts were prepared from transgenic plants and the presence of immunoglobulin heavy chain was detected using a murine immunoglobulin γ1 heavy chain specific capture ELISA. Representative data are shown in Figure 4.2 for plants expressing IgG, mγ1 and mIgG. In the IgG transgenic plant, Guy's 13 heavy chain was detected in plant extracts, and no difference was observed in the presence or absence of 1 % NP40 for membrane solubilisation. No heavy chain was detected in the non-transformed (WT) control plant. The results for two mγ1 and mIgG plants are shown. Although some heavy chain was detectable when plant extracts were prepared without detergent (-NP40) in these plants, there was a marked increase in heavy chain yield when membranes were solubilised (+NP40), suggesting that the majority of the heavy chain is associated with membranes in the plant cell.

Functional antibody was detected using an antigen specific ELISA, where streptococcal antigen I/II was coated on the ELISA wells, and detection of bound antibody was by a murine immunoglobulin γ1 heavy chain specific antiserum. The results are shown in Figure 4.3. Antigen specific Guy's 13 antibody was detected in the IgG plant and the

presence or absence of NP40 in the extraction buffer again had no effect. In plants co-expressing both the mγ1 and κ chains (i.e. mIgG plants), the antibody was also correctly assembled. As with the heavy chain specific ELISA (Figure 4.2), in mIgG plants, antibody was detected at a level 2-3 dilution steps higher in the presence of NP40, indicating that membrane solubilisation was necessary to release the accumulated antibody. The titration curves for IgG activity and heavy chain expression (Figure 4.2) were similar in these plants, indicating that there was little free heavy chain present and that most was assembled into functional antibody. No antigen binding was detected in extracts from non-transformed plants, or plants expressing mγ1 alone, as binding to native antigen requires both heavy and light chains.

4.2 Protoplast analysis

The ELISA data supported the hypothesis that mγ1 and mIgG were associated with membranes. We have investigated the expression of these recombinant molecules using protoplasts, to a) confirm the absence of antibody secretion, and b) to demonstrate that the membrane retention sequence results in protein retention in the plasma membrane.

Protoplasts were prepared from transgenic and control plants. The overnight protoplast culture supernatants, and solubilised membrane preparations were analysed by ELISA for the presence of immunoglobulin heavy chain (Figure 4.4).

There was little or no detectable immunoglobulin heavy chain in the membrane preparations from transgenic plants expressing the soluble form of IgG only (IgG plants), although there were detectable levels of secreted antibody in the culture supernatant. In contrast, the recombinant heavy chain was detectable in mγ1 plants in the ultracentrifuged membrane pellet resuspended in buffer containing NP40, but not the ultracentrifuged supernatant from the cell wall digestion buffer, which suggests that recombinant mγ1 is not secreted. Similar results were found for mIgG plants, although there were low levels of functional antibody detected in the supernatant. As before, the levels of mγ1detection were similar between mγ1 and mIgG plants. The data for the positive control (Guy's 13 IgG from murine hybridoma culture supernatant) and a negative control (WT) plant are also shown.

4.2.1 Immunofluorescent staining of protoplasts

Immunofluorescent staining with FITC labeled specific antisera was used to demonstrate the presence or absence of the recombinant proteins associated with the cell membrane of transgenic plant cells. For these studies, protoplasts were maintained as live cells. As controls, protoplasts were prepared from non-transformed (WT) plants as well as plants expressing irrelevant recombinant proteins.

Figure 4.5 shows the autofluorescence from protoplasts due to chlorophyll (Panel 1), from a control plant, transformed with an irrelevant protein. The autofluorescence is red and confined to the chloroplasts. Some of the cells appeared misshapen cells, but the majority were intact and apparently healthy. There was no FITC staining after incubation with FITC labeled anti-rabbit IgG antiserum (Panel 2) demonstrating the absence of cross reactive membrane proteins. Panels 3 and 4 show protoplasts from the same plant labeled with antibodies specific for the constitutive plasma membrane H^+-ATPase (De Witt *et al.*, 1996), which is a positive control plasma membrane marker protein. The bright green staining is evident, demonstrating the peripheral distribution of this protein.

For immunoglobulin heavy chain detection, protoplasts were probed with an FITC labeled anti-mouse gamma chain antiserum (Figures 4.6 and 4.7). Cells from wild-type plants (WT) were autofluorescent (Figure 4.6, Panels 1 and 2) but did not stain with FITC labeled anti-mouse antiserum (Figure 4.6, Panel 3). Approximately 1 in 30 protoplasts from non-transformed plants showed only weak fluorescence. In contrast, cells from mγ1 plants demonstrated bright surface staining (Figure 4.7, Panel 1) which is distinct from the chloroplasts and has a more circumferential localisation (Figure 4.7, Panels 2 and 3). The proportion of fluorescing cells in the mγ1 leaf preparations varied between 10 to 40 %. In the most highly fluorescing preparations, approximately 20 % of the cells were brightly fluorescent, a further 20 % had moderate fluorescence and the remainder emitted minimal or no fluorescent signals. The fluorescent pattern associated with protoplasts expressing mγ1 was similar to that seen when protoplasts were incubated with antiserum to plasma membrane H^+-ATPase, the control plasma membrane marker (Figure 4.5, Panels 3 and 4).

Membrane associated antibody was also demonstrated by immunofluorescence detection of light and heavy chain expression in protoplasts from transgenic plants. Protoplasts were labeled with either FITC labeled anti-mouse kappa chain, or FITC labeled anti-mouse

gamma chain antisera. With the anti-kappa chain reagent, there was no specific staining of cells from non-transformed plants (WT), nor from those only expressing the mγ1 chain (Figure 4.8, Panels 1 to 4). This demonstrates the specificity of the anti-kappa chain antiserum, and the absence of cross-reactivity with Ig heavy chain. mγ1 cells were positively stained (as before) for anti-gamma chain (Figure 4.9, Panels 3 and 4). Protoplasts from plants co-expressing mγ1 and kappa demonstrated surface staining both with anti-kappa (Figure 4.8, Panels 5 and 6) and anti-gamma (Figure 4.9, Panels 1 and 2) antisera. This confirms the presence of surface immunoglobulin heavy chain in mIgG plants, as well as the co-localisation with kappa chain at the same site. The frequency of brightly stained protoplasts from mIgG plants was similar to that found with mγ1 plants stained with anti-gamma chain antiserum.

4.3 Expression of the chemokine receptor CCR-5

The demonstration that the Ig membrane anchoring sequence was functional in plants was the first of its kind for mammalian targeting sequences. We were interested to extend these observations to other mammalian membrane proteins, in order to establish the potential for mammalian membrane protein expression in plants in general.

CCR-5 is a chemokine receptor, and a member of the 7-transmembrane segment G-protein coupled receptor (GPCR) family (Samson *et al.*, 1996)). These receptors are found primarily on the surface of T cells and macrophages in the immune system. Thus the aim was to express a recombinant CCR-5 protein in transgenic plants, and to determine if the protein was targeted to the cell membrane.

A cDNA clone encoding human CCR-5 was kindly provided by Professor T. Lehner. This sequence, along with its native leader sequence was cloned into the pMON 530 plant expression vector (used for all the transgenic plants studied) under the control of the CaMV 35S promoter. Transgenic plants were generated by Dr. Pascal Drake in our laboratory.

4.3.1 RT-PCR confirmation of transgenic plants.

Putative transgenic plants expressing CCR-5 were screened by RT-PCR using specific CCR-5 oligonucleotides (sequences shown in Appendix). A major band of approximately 1000 bp, the expected size, was detected in 3 out of 4 plants tested (Figure 4.10). In each case the PCR was performed using cDNA (+) or the parent RNA (-) [i.e. with no reverse

transcriptase – as described in Materials and Methods] to confirm the absence of contaminating genomic DNA in the RNA preparations. 2 control plants were included, one non-transformed wild type plant, and one plant transgenic for an irrelevant protein.

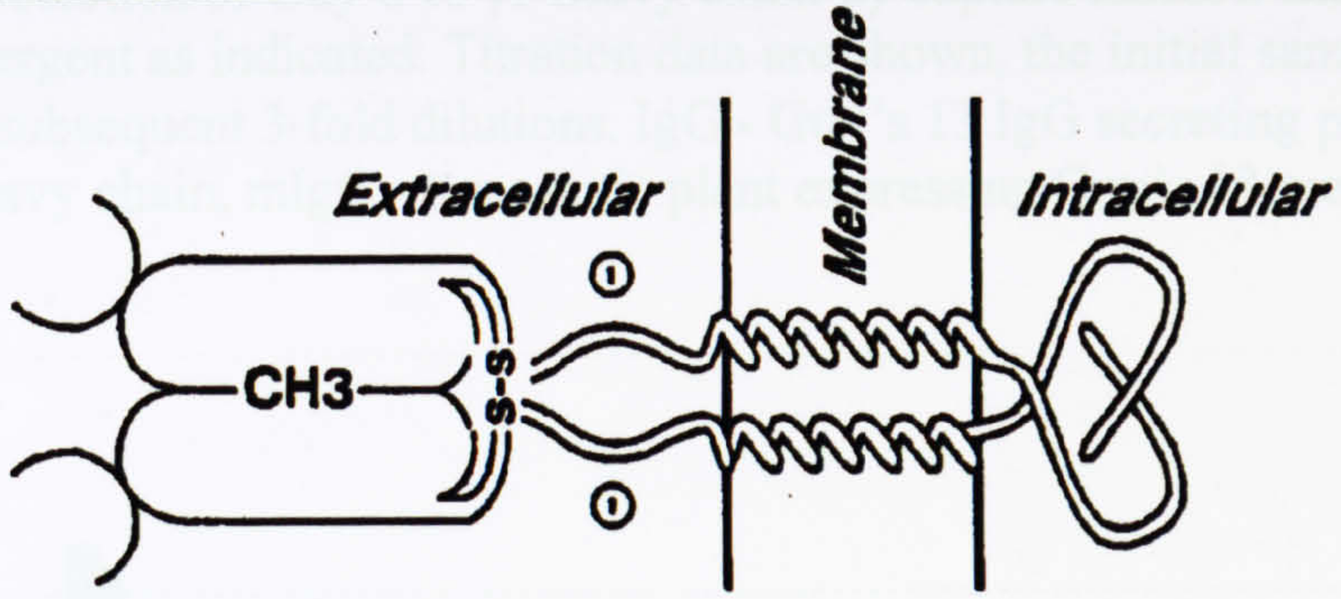
4.3.2 ELISA and Western blot analysis of transgenic plants.

Transgenic plants identified by RT-PCR were used for analysis by ELISA and Western blot. As for the detection of immunoglobulin chain, crude leaf extracts were prepared in tris buffered saline, with protease inhibitor (leupeptin), but no recombinant protein was detectable by either a CCR-5 capture ELISA or immunoblotting using a monkey anti-CCR-5 antiserum (results not shown). Unlike the mγ1 and mIgG expressing plants, addition of detergents – SDS, Triton X-100, NP-40 to the extraction buffer made no difference and we were unable to detect CCR-5 protein by these immunological techniques.

4.3.3 Protoplast immunofluorescence

Immunofluorescent staining of protoplasts was performed using the CCR-5 specific monkey antiserum kindly provided by Dr. Lesley Bergmeier. Bound antibody was detected using FITC labeled anti-monkey antiserum.

As with the FITC labeled rabbit antisera used in the membrane Ig experiments, approximately 1 in 30 protoplasts from non-transformed plants (WT) showed weak background fluorescence (not shown). In contrast, cells from the CCR-5 plants demonstrated bright surface staining (Figure 4.11). The numbers of fluorescing cells in each preparation varied between 20 to 40 %. The fluorescent pattern associated with protoplasts expressing the CCR-5 molecule was similar to that observed with protoplasts incubated with antiserum to plasma membrane H^+-ATPase, and membrane associated IgG as described in the previous section.



GGGCTGCAACTGGACGAGACCTGTGCTGAGGCCCAGGACGGGGAGCTGGA CGGGCTCTGGACGACCATCACCATC
G L Q L D E T C A E A Q D G E L D G L W T T I T I

Hinge *----------Trans--------*

TTCATCAGCCTGTTCCTGCTCAGCGTGTGCTACAGCGCTGCTGTCACACTCTTC AAGGTAAAGTGGATCTTCTCC
F I S L F L L S V C Y S A A V T L F K V K W I F S

--Membrane----------

TCGGTGGTGGAGCTGAAGCAGACACTGGTTCCTGAATACAAGAACATGATTGGGCAAGCGCCC TAGAATTC
S V V E L K Q T L V P E Y K N M I G Q A P

Intracellular

Figure 4.1: **Murine Ig Membrane Spanning Region.** Diagrammatic representation of the C-terminus of mγ1 and predicted amino acid sequence of Guy's 13 mγ1 heavy chain. The functional regions are indicated.

Figure 4.2: Detection of Guy's 13 γ1 heavy chain by capture ELISA. Extracts were prepared in the presence or absence of 1% NP40 detergent as indicated. Titration data are shown, the initial sample was a 3-fold dilution of the crude plant extract (black), with subsequent 3-fold dilutions. IgG - Guy's 13 IgG secreting plant, WT - non-transformed plant, mγ1 - Guy's 13 membrane heavy chain, mIgG - transgenic plant expressing Guy's 13 membrane heavy chain and Guy's 13 light chain.

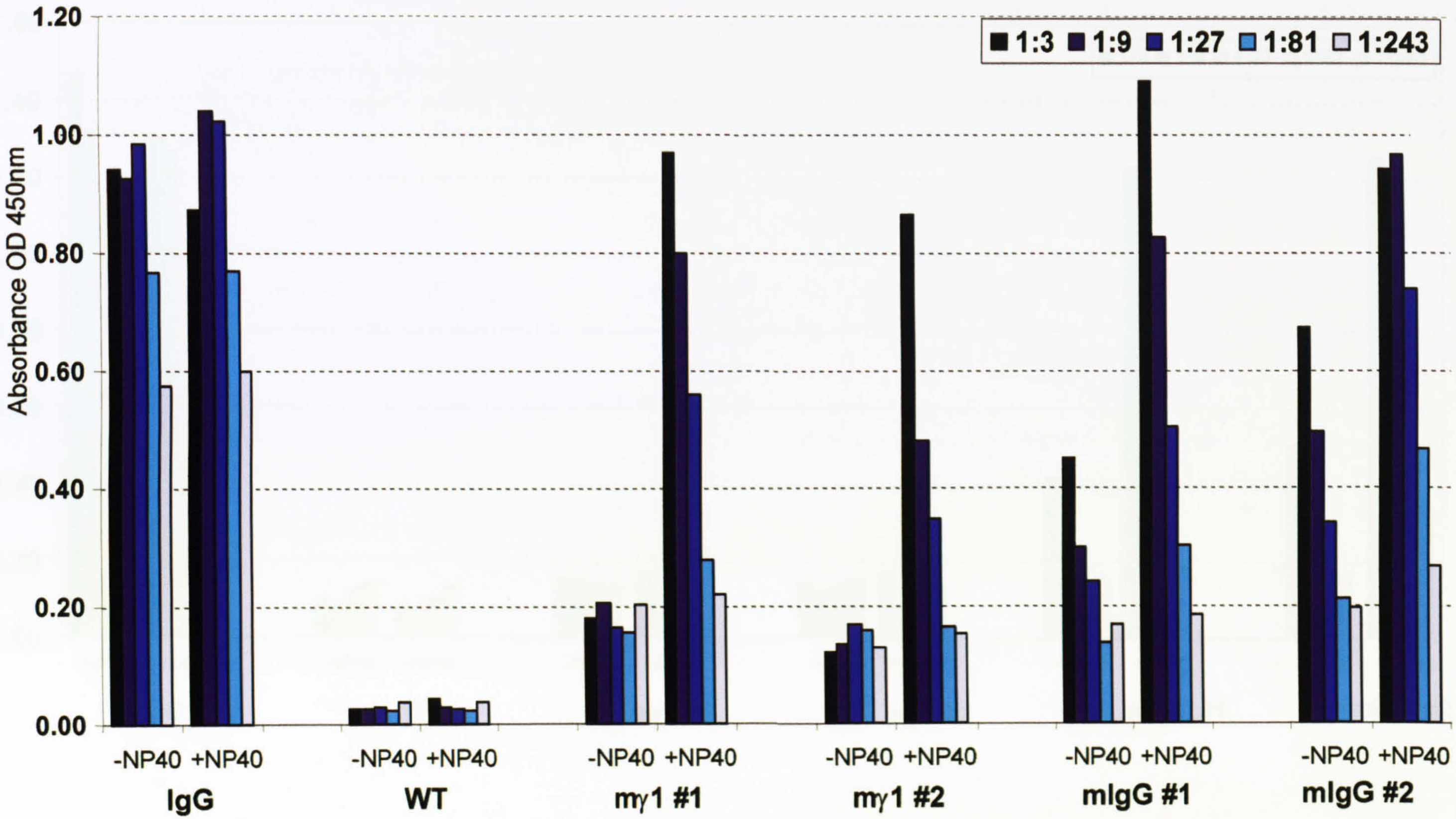


Figure 4.3: Detection of functional antibody by sandwich ELISA using streptococcal antigen I/II. Extracts were prepared in the presence or absence of 1% NP40 detergent as indicated. Titration data are shown, the initial sample was a 3-fold dilution of the crude plant extract (black), with subsequent 3-fold dilutions. IgG - Guy's 13 IgG secreting plant, WT - non-transformed plant, mγ1 - Guy's 13 membrane heavy chain, mIgG - transgenic plant expressing Guy's 13 membrane heavy chain and Guy's 13 light chain.

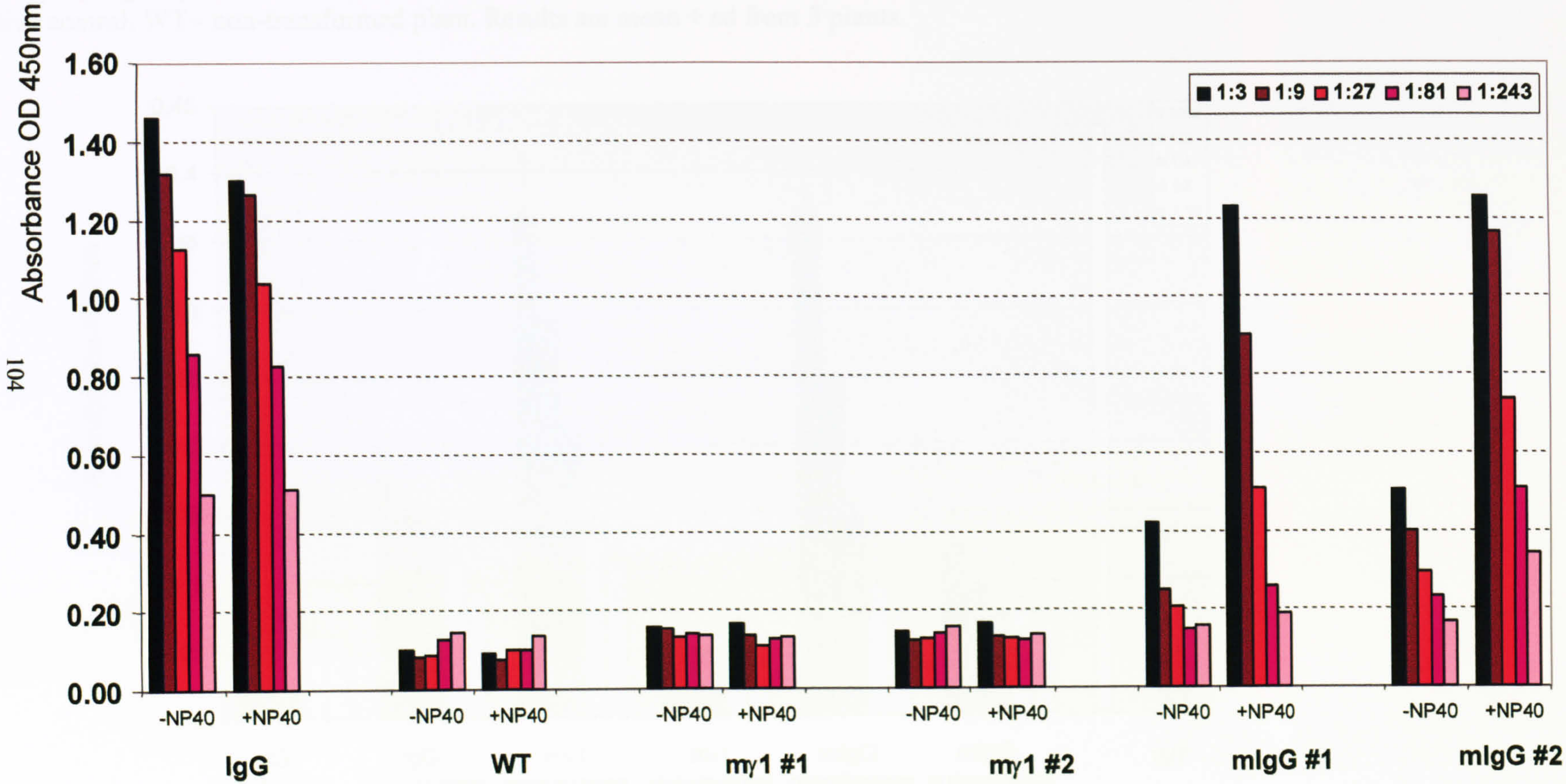
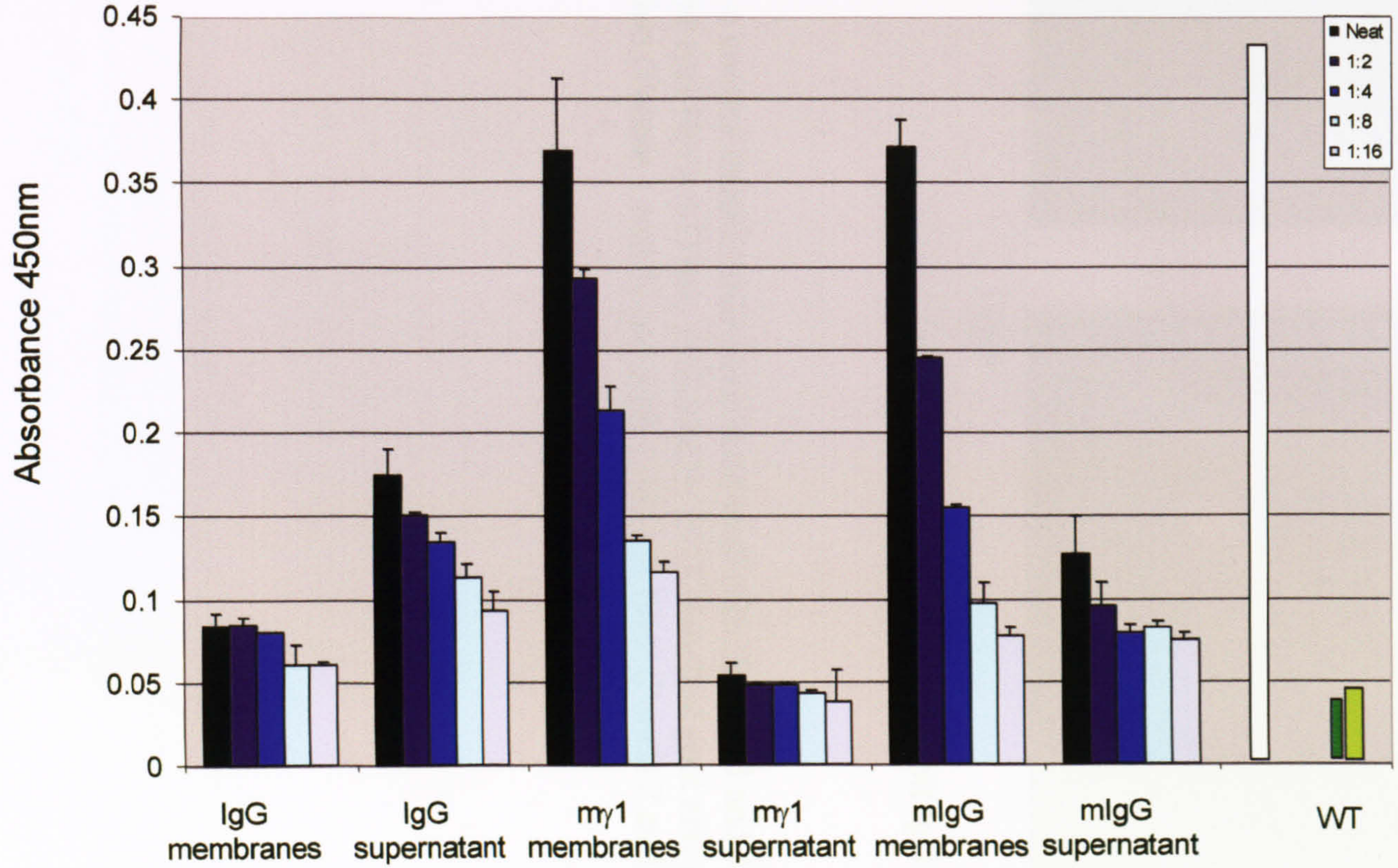


Figure 4.4: Detection of Guy's 13 mγ1 heavy chain in samples prepared from protoplasts or culture supernatants. The extracts were analysed neat and in 2-fold dilution steps. IgG - Guy's 13 IgG secreting plant, mg1 - Guy's 13 membrane heavy chain, mIgG - transgenic plant expressing Guy's 13 membrane heavy chain and Guy's 13 light chain, Guy's 13 IgG - murine hybridoma culture supernatant positive control, WT - non-transformed plant. Results are mean + sd from 3 plants.



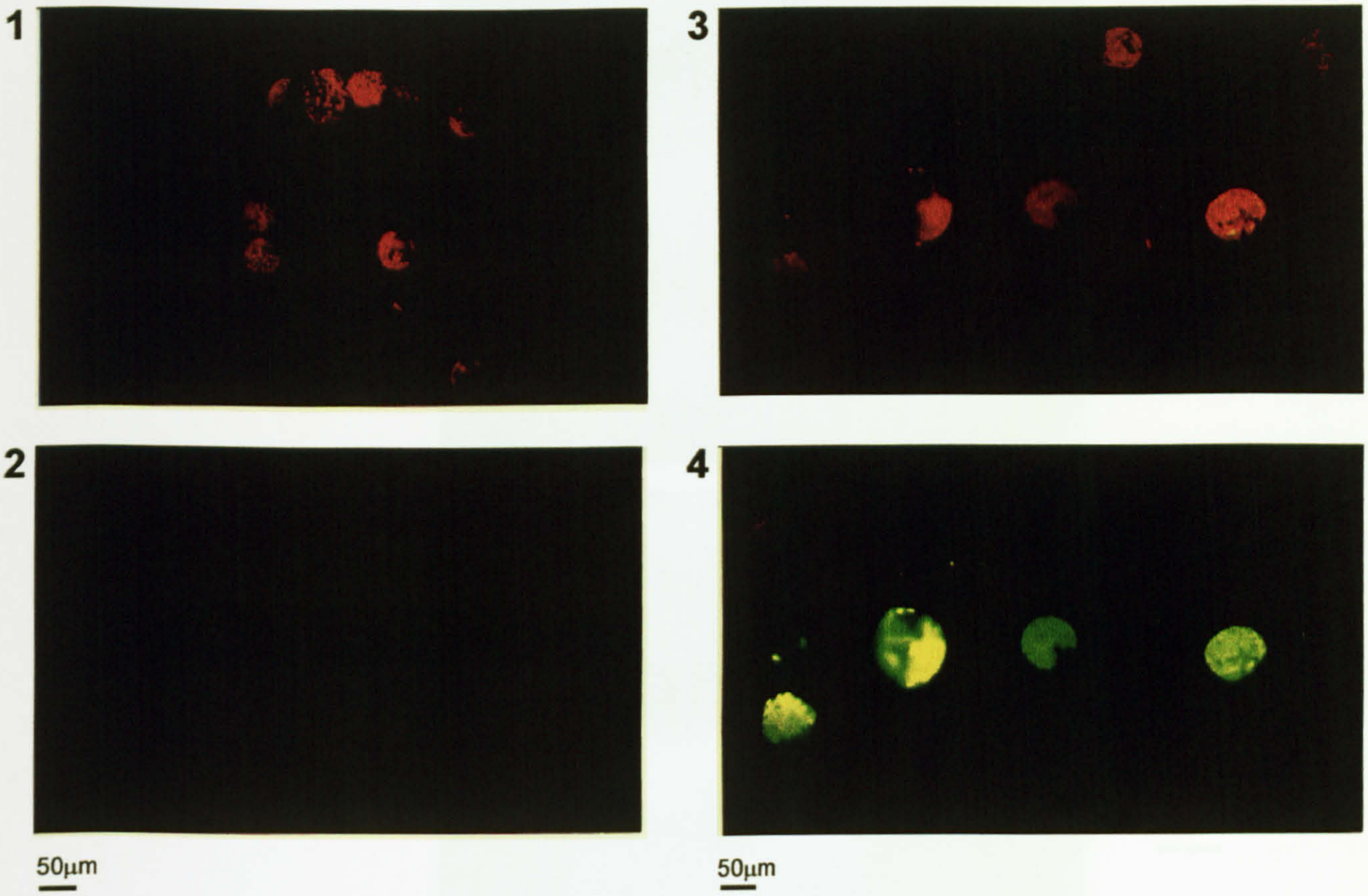
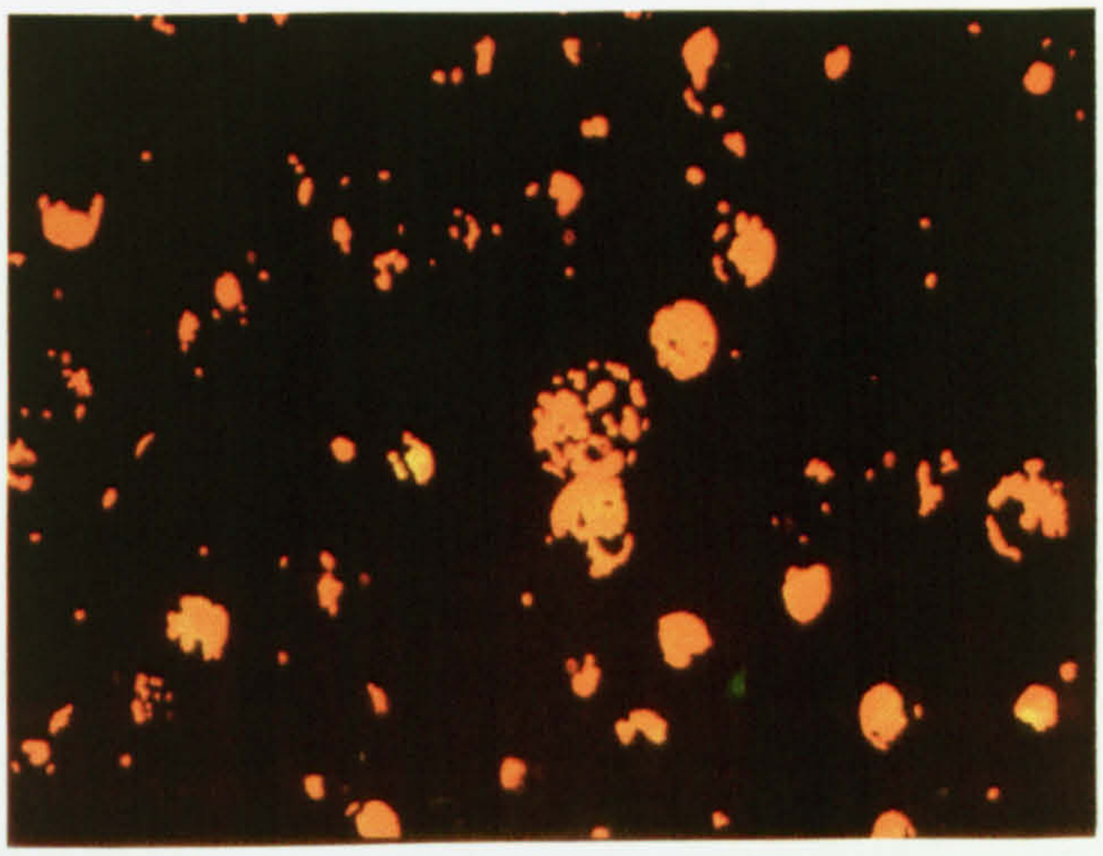
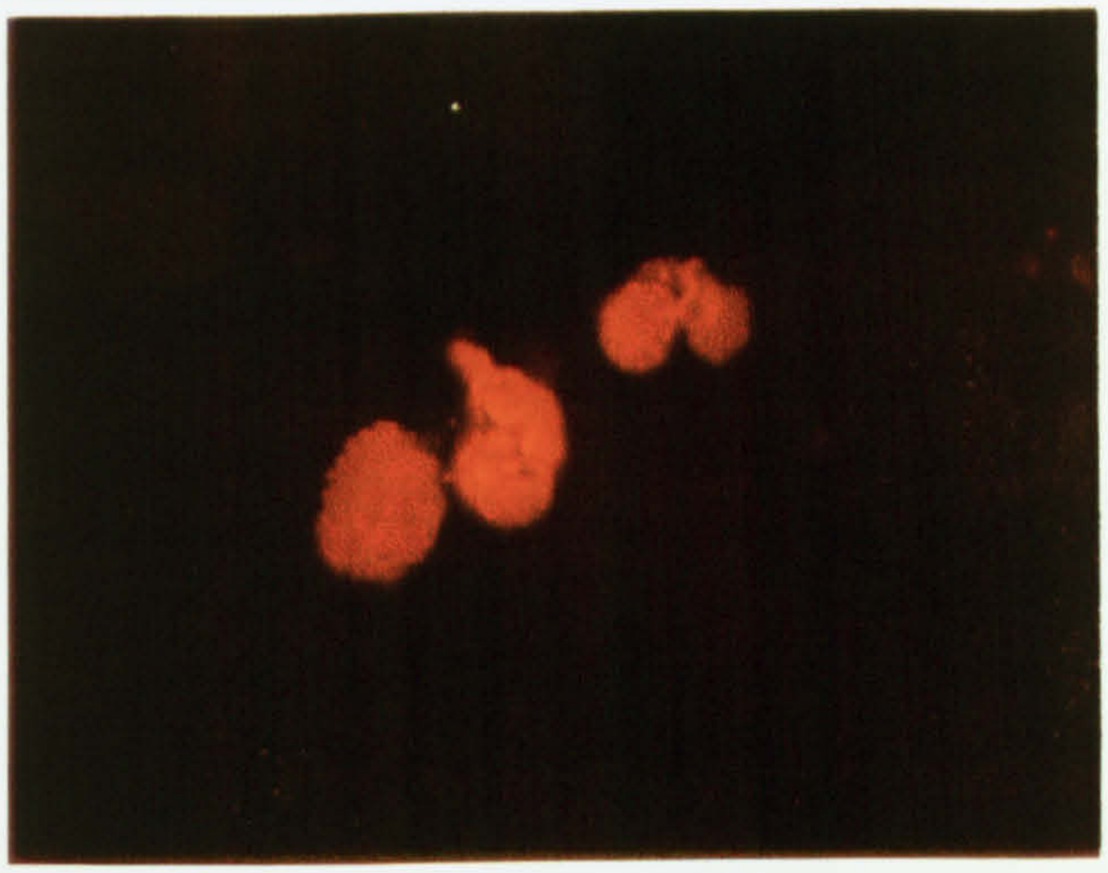


Figure 4.5: Protoplasts from a control transformed plant incubated with (Panels 3 and 4) or without (Panels 1 and 2) rabbit anti-H(+)-ATPase, followed by FITC-labelled goat anti-rabbit IgG antiserum. UV illumination (Panels 1 and 3) and with a 460 nm filter (Panels 2 and 4) .

1



2



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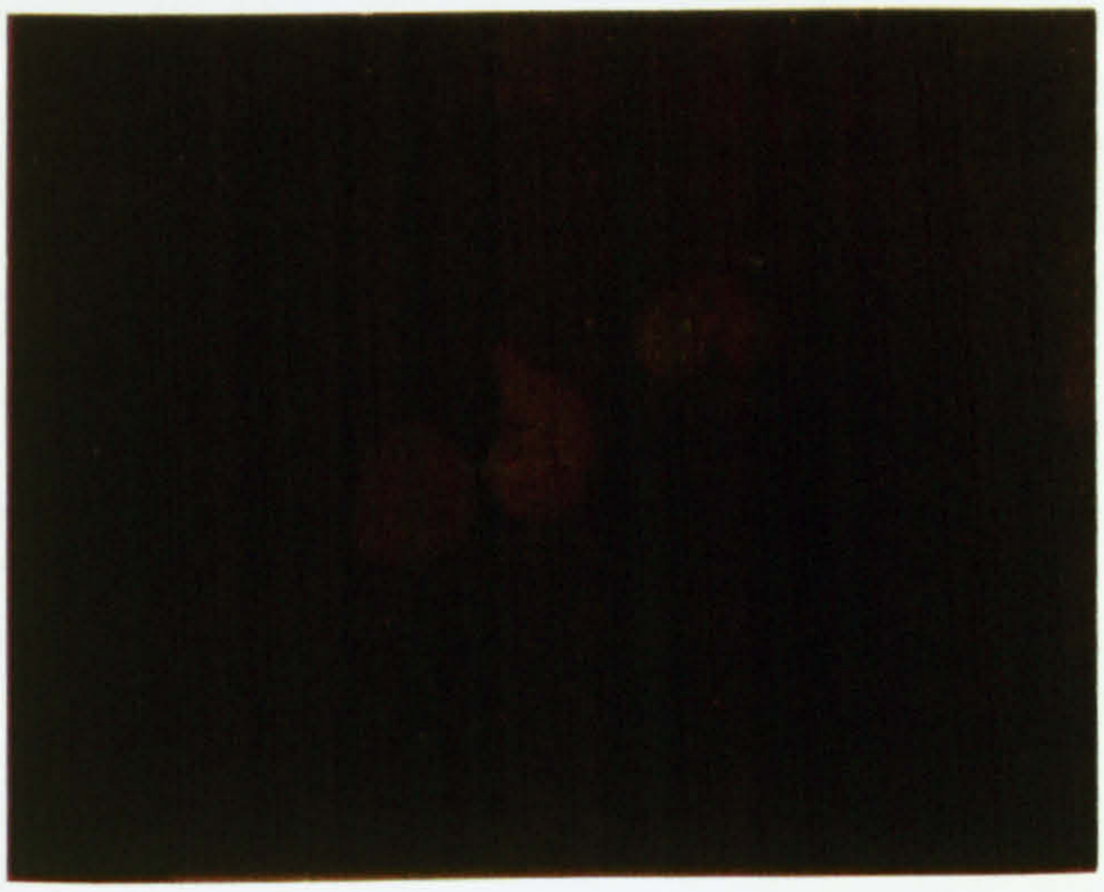
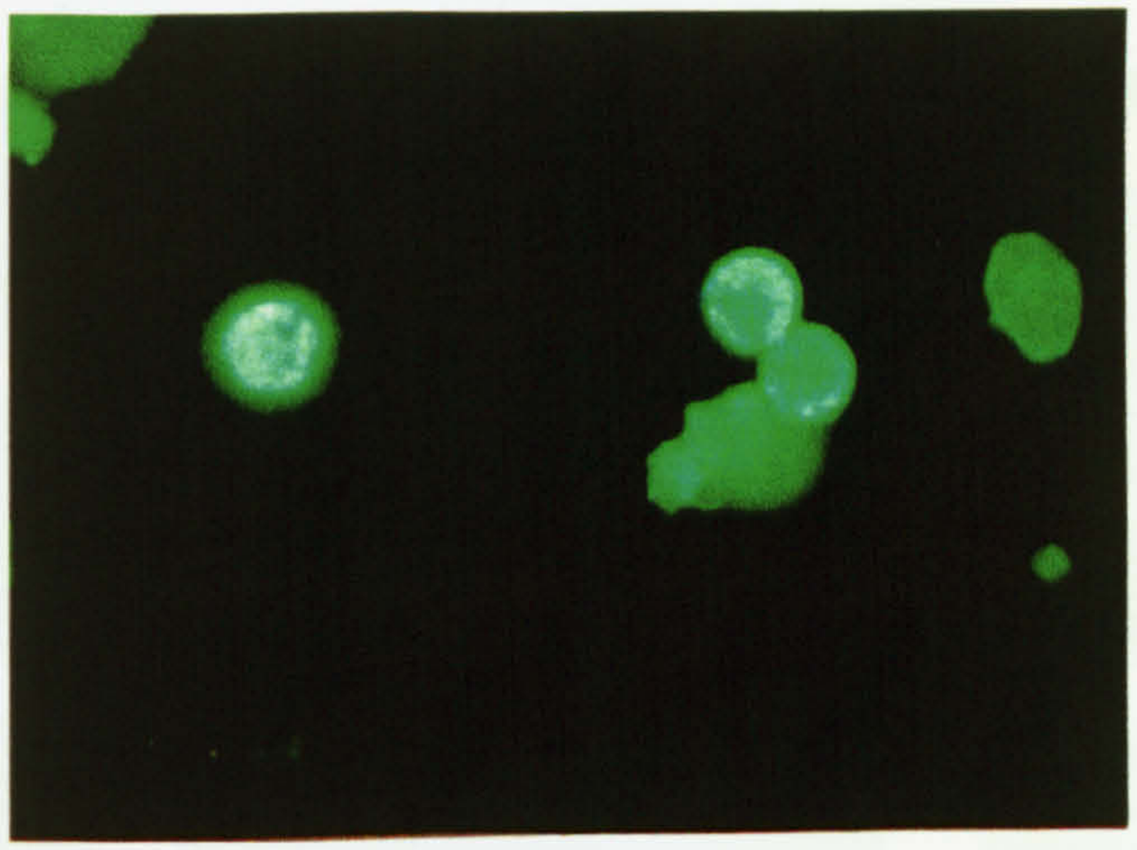
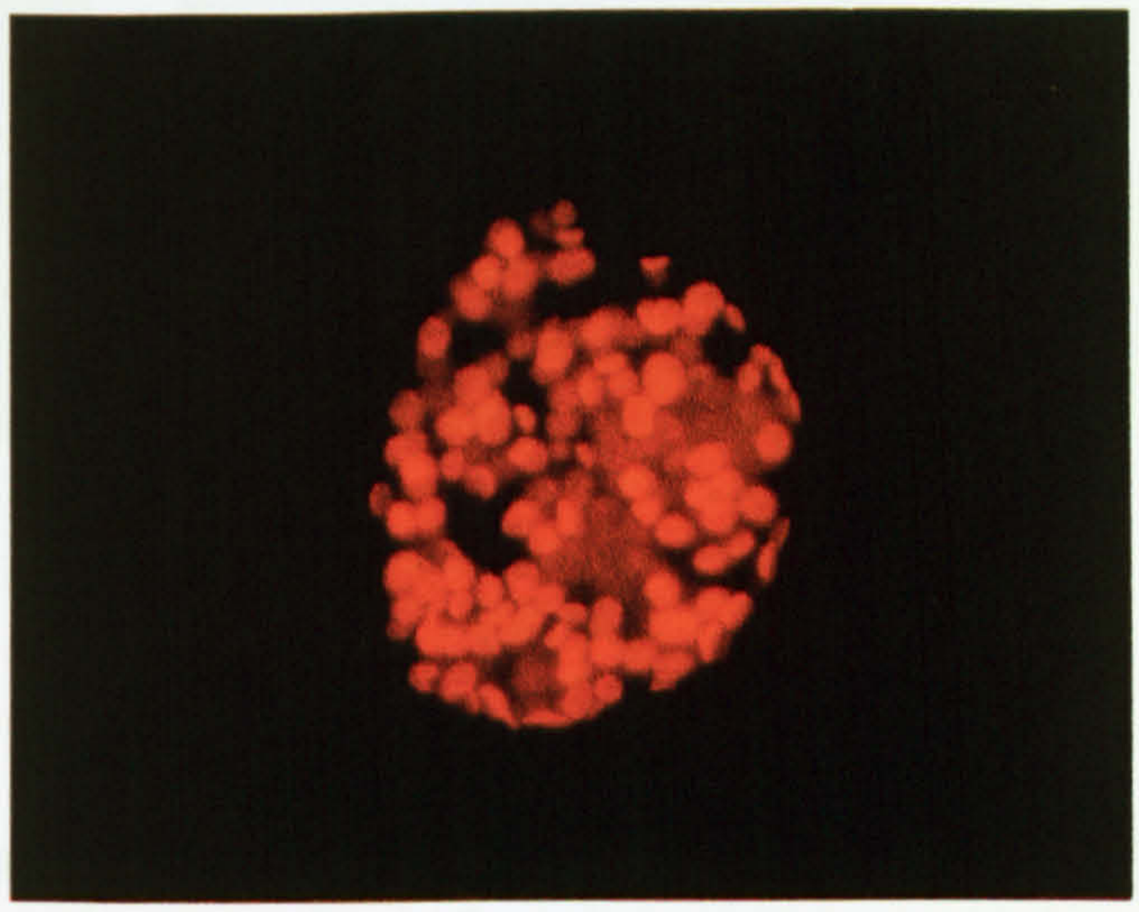


Figure 4.6 : Immunofluorescent staining of non-transformed protoplasts. Non-transformed plant (WT) cells incubated with FITC-labelled goat anti-mouse γ1 antiserum showing auto-immunofluorescence under UV illumination (Panels 1 and 2) and with a 460 nm filter (Panel 3).





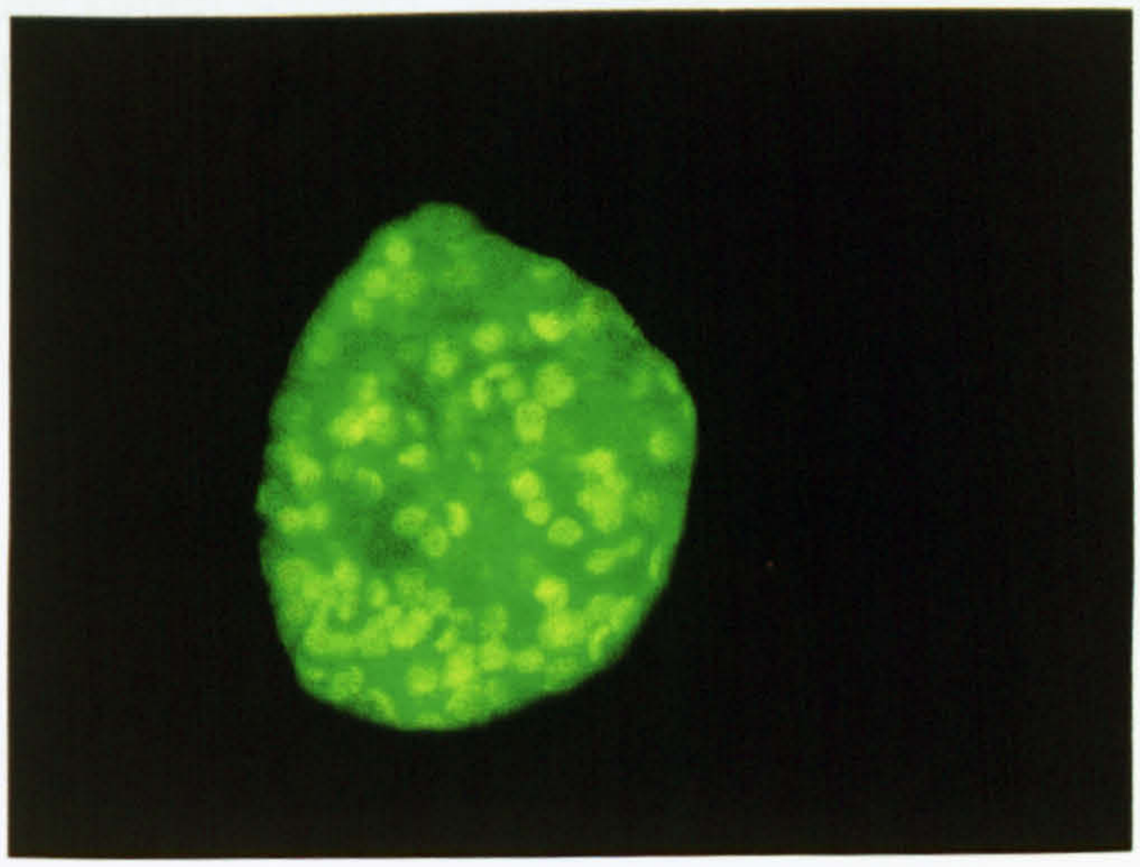


Figure 4.7: Immunofluorescent staining of transgenic protoplasts from mγ1 transformed plants. Protoplasts from transgenic plants expressing mγ1 chain, incubated with FITC-labelled goat anti-mouse γ1 antiserum under UV illumination (Panel 2) and 460 nm filter (Panels 1 and 3).

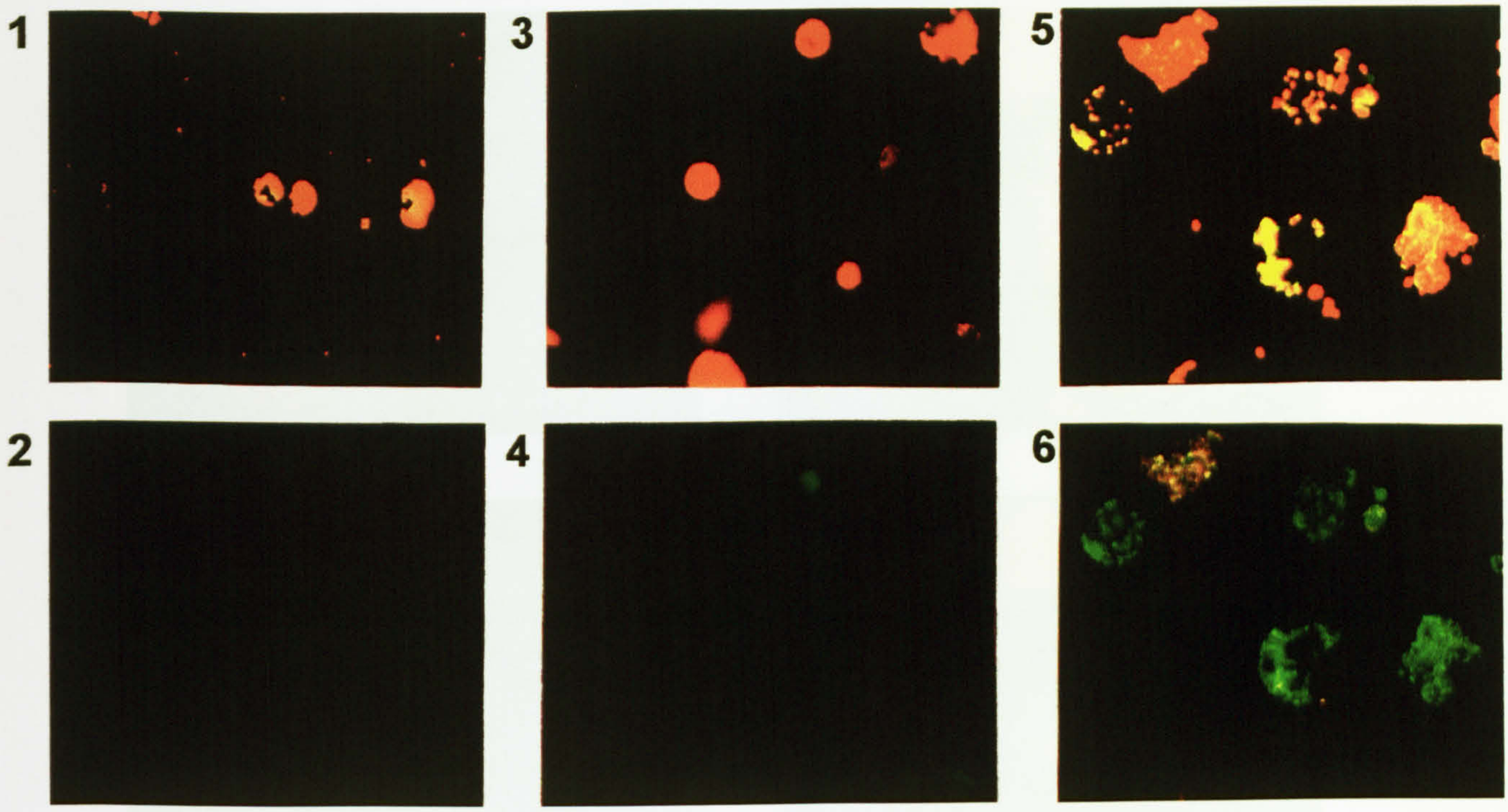


Figure 4.8: Immunofluorescent staining of non-transformed and transgenic protoplasts with FITC labeled goat anti-mouse kappa antisera. Samples are shown with UV illumination, with (Lower) or without (Upper) a 460 nm filter. WT cells (Panels 1 and 2), mγ1 cells (Panels 3 and 4) and mIgG cells (Panels 5 and 6).

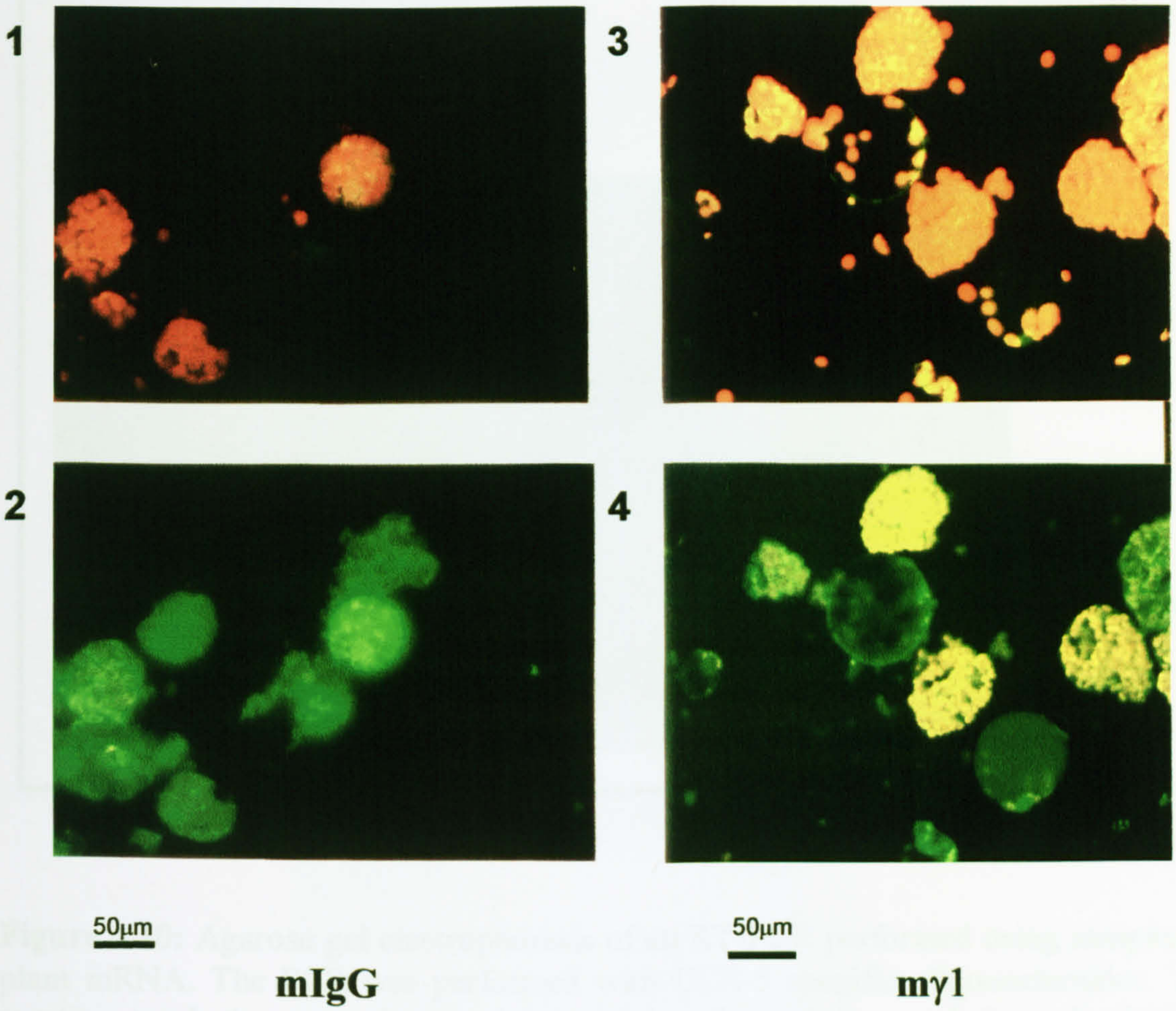


Figure 4.9: Immunofluorescent staining of transgenic protoplasts with FITC labeled goat anti-mouse γ1 antisera. Samples are shown with UV illumination, with (Lower) or without (Upper) 460 nm filter. mIgG cells (Panels 1 and 2), mγ1 cells (Panels 3 and 4).

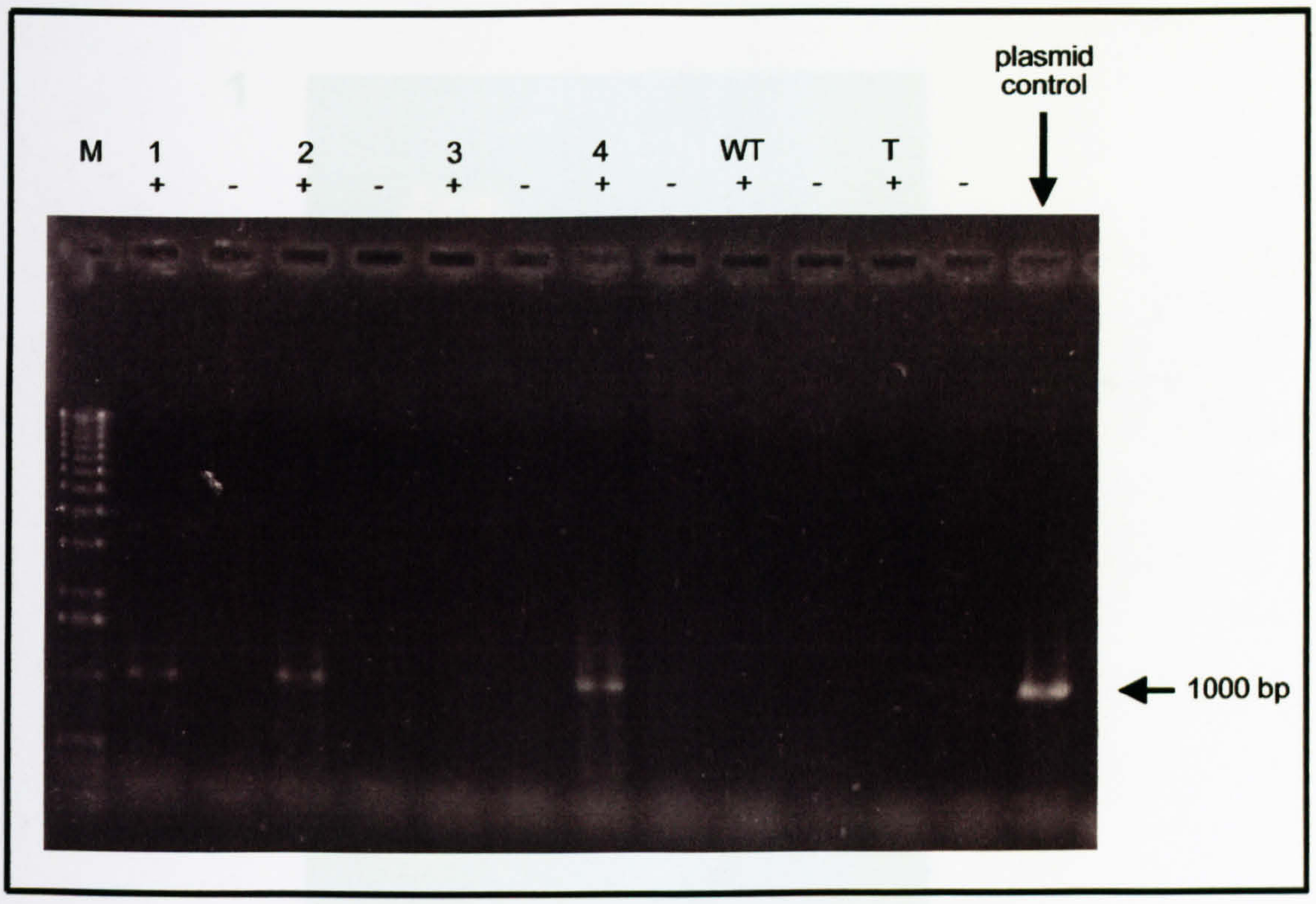


Figure 4.10: Agarose gel electrophoresis of an RT-PCR performed using samples of plant mRNA. The PCR was performed with CCR-5 specific oligonucleotides. The reaction products were visualised by staining the gel in a solution of ethidium bromide and observing under UV light. Samples are; 1 – 4, putative transgenic plants expressing CCR-5; WT - non-transformed plant; T - transgenic plant expressing an irrelevant recombinant protein; Plasmid control - pBluescript containing CCR-5 gene insert. "+" indicates cDNA was used as template for the RNA, "-" indicates the template was purified RNA, before reverse transcription. DNA size markers (lane M) are indicated in kilobases.

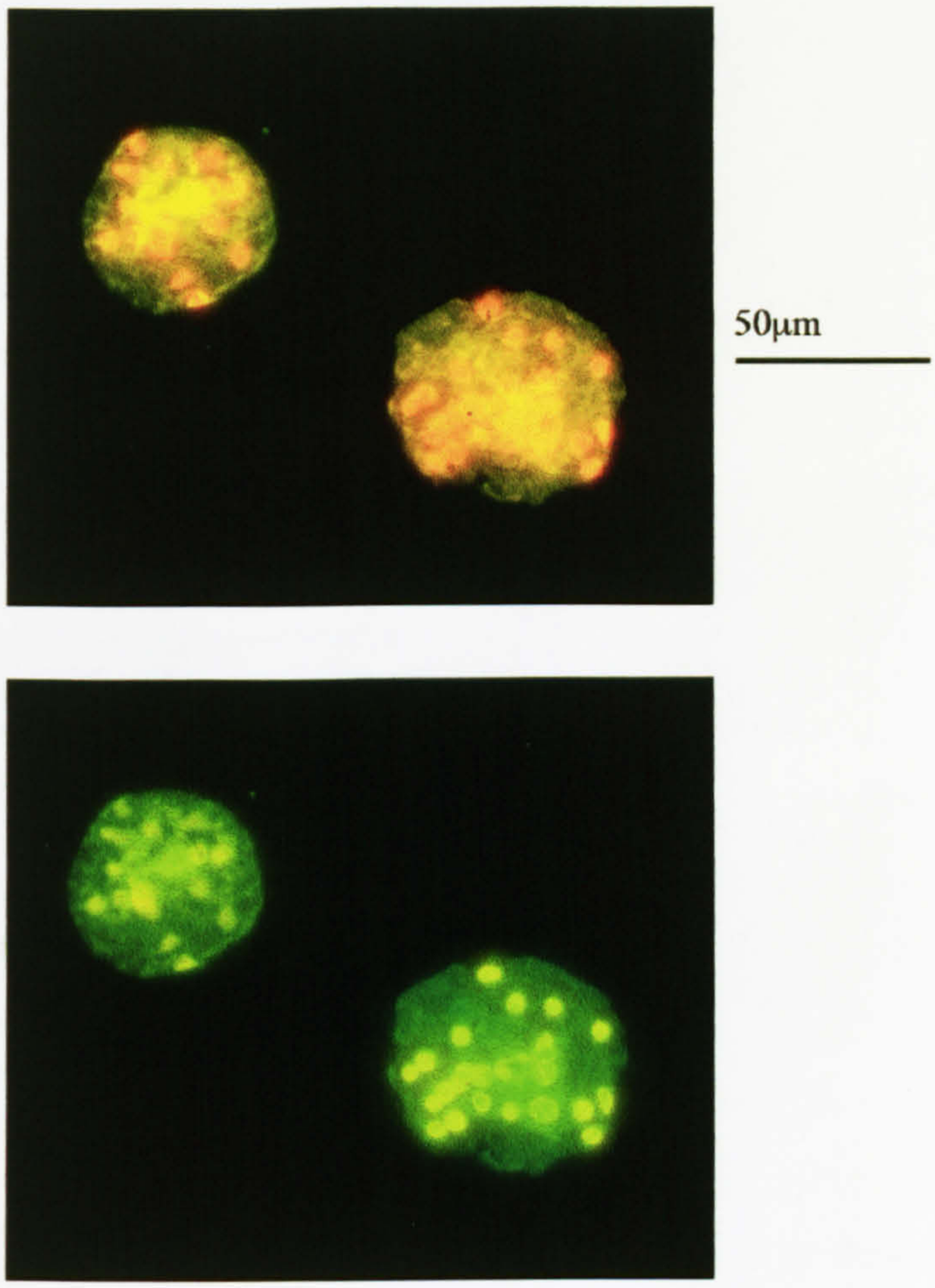


Figure 4.11: Immunofluorescent staining of protoplasts from transgenic plants expressing CCR-5. Incubation was with a monkey anti-CCR-5 antiserum, followed by a FITC labeled anti-monkey IgG antiserum. Cells were viewed under UV illumination, without (Panel 1) and with 460 nm filter (Panel 2).

4.4 Discussion

The expression of antibodies in plants has many potential applications, including human health that is likely to require extraction and purification, as well as plant health where targeting to sub-cellular compartments might be required. In the latter case, it has already been shown that cytosolic single chain Fv fragments can protect against viral infection (Tavladoraki *et al.*, 1993). We have already shown that the default pathway for recombinant IgG in plants is secretion, once the immunoglobulin chains have been targeted to the endomembrane system with a leader peptide. In this chapter, we have investigated the targeting of full-length antibodies to the plant cell membrane, using the native murine immunoglobulin transmembrane sequence with a view to potential applications beyond antibody production for immunotherapy.

The murine immunoglobulin transmembrane sequence encodes a short extracellular region, a hydrophobic transmembrane sequence comprising 26 amino acid residues and a hydrophilic 28 residue intracellular domain (Tyler *et al.*, 1982). Here we have shown that immunoglobulin heavy chain bearing this sequence is retained by the plant plasma membrane. Using a simple procedure, soluble recombinant protein could not be detected unless extraction was performed in the presence of a non-ionic detergent - NP40. It is most likely that the recombinant heavy chain is retained in the cell membrane as the protein was targeted to the secretory pathway by a signal sequence, and also because the heavy chain was readily detected by immunofluorescence of protoplasts. Furthermore, the mγ1 assembled correctly with the immunoglobulin light chain which does not possess a transmembrane sequence but is also targeted for secretion using a related signal sequence. Assembly of light and heavy chain occurs in the ER in plants (Nuttall *et al.*, 2002) and mammals and they emerge at the cell membrane as fully assembled immunoglobulin. We have demonstrated in the mIgG plants that the kappa and gamma chains are co-localised to the cell membrane using immunofluorescence and that they remain correctly assembled at this site as antibody, using a functional antigen specific ELISA. The staining pattern of both mγ1 and mIgG at the plasma membrane was similar to that seen for a native plasma membrane component [H(+)-ATPase pump], indicating that these recombinant proteins are being targeted to the plasma membrane in a normal manner. Our studies do not however exclude the possibility that the recombinant antibodies might also be distributed elsewhere in the plant cell and further investigation of the fine cellular distribution of mγ1 and mIgG

needs to be studied by sub-cellular fractionation studies or immuno-electron microscopy. Recently it has been demonstrated that engineered immunoglobulins originally targeted for secretion, can also be retained in the ER and the vacuole (Hadlington *et al.*, 2003). It remains to be determined if the murine immunoglobulin transmembrane sequence possesses signals that are specifically recognised by plants, but the accumulation of a significant proportion of the recombinant immunoglobulin heavy chain and antibody at the cell membrane suggests a default secretory pathway to the cell surface.

The mIgG appears to be more readily dissociated from plant cell membranes than mγ1 alone as shown by the detection of antibodies in plant extracts without NP-40. This may be due to over accumulation of IgG in the extracellular compartment, or it is possible that the additional burden of the membrane anchored recombinant antibody destabilises the cell membrane. However, protoplasts prepared from these plants appeared no less robust than others prepared from non-transformed plants. The functional ELISA of extracts from mγ1 and mIgG plants (Figure 4.2) suggests a higher level of accumulation of assembled IgG compared with the heavy chain clone by 2 dilution steps (approximately 10-fold). This is consistent with previous findings (Hein *et al.*, 1991) that suggested that Ig heavy chains are stabilised in plants by assembly into antibody. Not all protoplasts expressed the mγ1 heavy chain and this is consistent with the finding that the 35S CaMV promotor does not direct expression in all cells all of the time (Barnes, 1990; Benfey & Chua, 1989). It will be of interest to determine which cell types express the recombinant immunoglobulin chains and at which stages of development.

Many membrane proteins have proved difficult to express in recombinant systems. Membrane proteins have different forms (Figure 3, next page) and in the second part of this study we have extended our findings on membrane immunoglobulins by investigating a different kind of mammalian membrane protein, the chemokine receptor CCR-5. The CCR-5 receptor is found primarily on T cells and macrophages. It is a member of the 7-transmembrane segment G-protein coupled receptor (GPCR) family of proteins and therefore unrelated to the immunoglobulin supergene family of proteins. Although 7-transmembrane proteins have a variety of different functions, they all have a highly conserved 3-dimensional structure (Muller, 2000). In all 7-transmembrane proteins, the portion within the lipid bilayer consists mainly of hydrophobic amino acids. These are usually arranged in an alpha helix, so that the polar –C=O and –NH groups at the peptide

bonds can interact with one another, rather than with their hydrophobic surroundings. Those parts of the polypeptide that project out from the bilayer usually have a high percentage of hydrophilic amino acids. In addition, those that project into the aqueous surroundings of the cell are mainly glycoproteins, with many hydrophilic sugar residues attached to the part of the polypeptide exposed at the surface of the cell. Whilst more than 150 different members of the 7-transmembrane domain protein family have been identified, all have the C-terminal end of the peptide facing the cytoplasm and the N-terminal end facing the extracellular fluid (Schoneberg *et al.*, 1999).

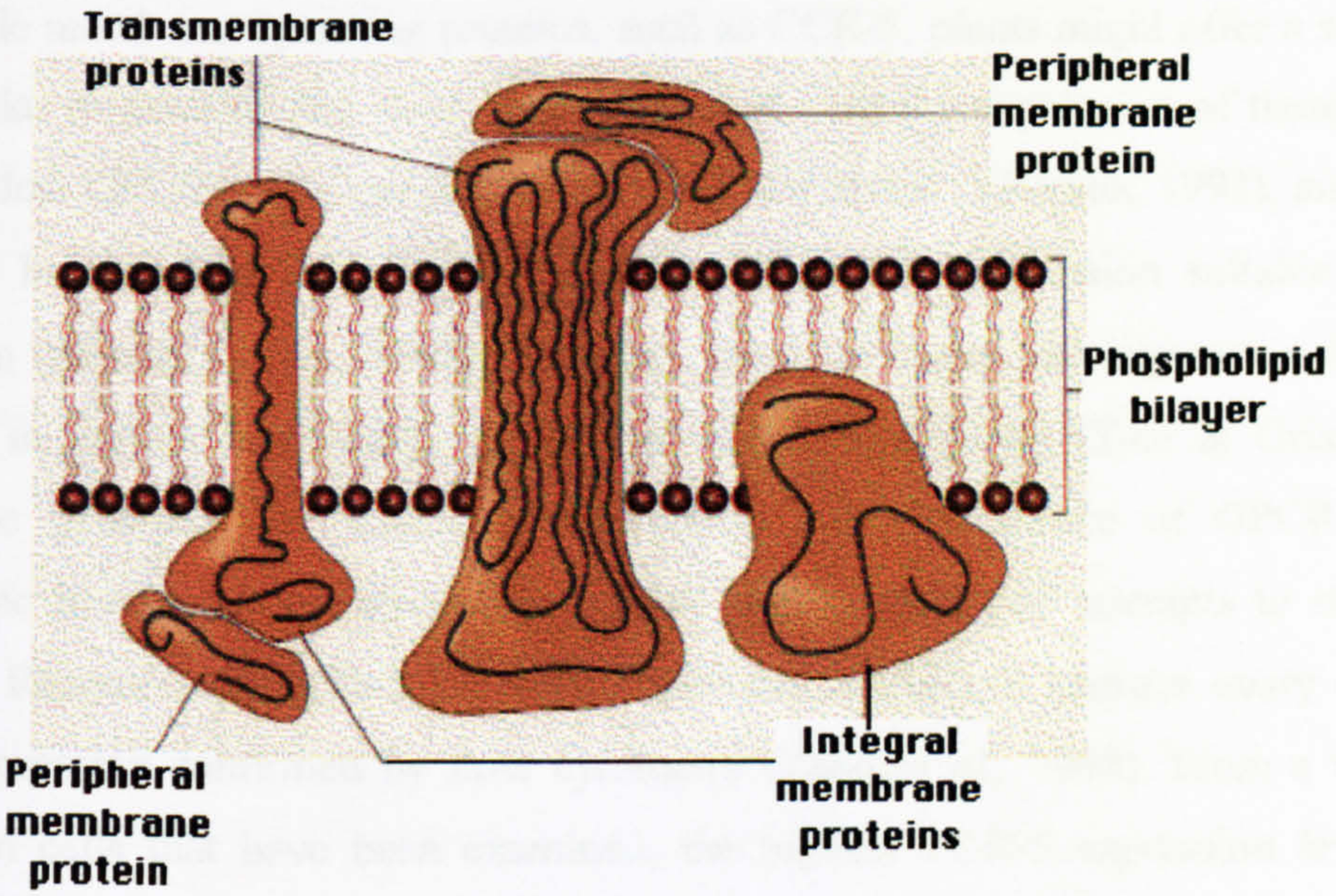


Figure 3: Schematic representation of membrane proteins.

The expression of CCR-5 protein could not be detected from crude transgenic plant extracts by ELISA or Western blot. We believe that this could be due to either very low levels of expression (below the limits of detection), or more likely that the protein was denatured and degraded during extraction. However we were able to detect CCR-5 mRNA by RT-PCR of transgenic plants. We have also demonstrated surface staining of plant protoplasts using an antibody specific for CCR-5, in a similar manner to that used for detecting membrane immunoglobulin. Further studies will be required to demonstrate the correct conformation of this recombinant protein and fidelity of function, but these

preliminary studies suggest that a 7-transmembrane mammalian protein may be treated and sequestered in plants in the same manner as in humans.

There are many potential applications for membrane protein expression in plants. In the case of antibodies, retention and accumulation of specific antibodies in an extracellular compartment might help to prevent infection by interfering with microbial invasion. There may also be applications related to the use of cell suspension cultures or protoplasts, for example in the large scale adsorption of antigens. Furthermore, plant antibodies that accumulate but are largely retained in the apoplasm may be useful in the area of phytoremediation and environmental clean-up.

For multiple membrane spanning proteins, such as CCR-5, plants might offer a solution to the particular difficulties that have been associated with the expression of these kinds of proteins. Most GPCRs are expressed naturally at low levels (Khorana, 1992), and thus far no system has resulted reproducibly in levels of protein expression suitable for their purification (Stanasila *et al.*, 1998). Bacterial, yeast, or insect cell expression of GPCRs can result in protein misfolding, aggregation and heterogeneity (Tate & Grisshammer, 1996). The generally low levels of expression and dependence of GPCRs on the hydrophobic intramembrane environment have also complicated attempts to study their structure. Recombinant CCR-5 has been expressed in Chinese hamster ovary cells, and surface expression confirmed by flow cytometry (Zhao *et al.*, 1998). From a variety of mammalian cells that have been examined, the highest CCR-5 expression levels were observed in canine thymocytes and human embryonic kidney cells (Mirzabekov *et al.*, 1999). It was proposed that scale-up of these cells could allow the purification of milligram amounts of the CCR-5 protein. None of these cell culture systems is straightforward, requiring sterility and expensive media. Our findings suggest that plants may be an alternative system.

The function of CCR-5 is to act as a receptor for binding chemokines, the end result of which is to recruit cells of the immune system to the site of tissue damage or disease (Raport *et al.*, 1996). HIV-1 takes advantage of the presence of these chemokine receptors to gain access to the cell via a fusion-mediated event (Alkhatib *et al.*, 1996). The identification of non-functional CCR-5 alleles in individuals (Zimmerman *et al.*, 1997) provided fresh ideas for prevention strategies, such as blockage or inhibition of receptor function by immunisation with portions of, or the entire CCR-5 to generate appropriate

antibodies (Bogers, unpublished). Recombinant CCR-5 protein might also be used in biochemical and structural studies, or for diagnostic assays and screening purposes.

An exciting possibility that is raised by our findings, is the potential for using mammalian membrane receptor proteins as targets that would initiate signalling mechanisms in plants. However, a considerable amount of further engineering would be required. For example, in addition to mIgG, the B-cell receptor complex involves two other proteins (alpha and beta chains) which each have a tyrosine-based activation motif in their cytoplasmic tails that triggers the signalling pathway in B cells. The G-protein signalling pathway is a highly conserved mechanism for transducing extracellular signals in a variety of organisms, and the superfamily of 7-transmembrane receptors is a central component of this pathway. The first 7-transmembrane receptor identified in higher plants was shown to be encoded by the gene GCR1 in *Arabidopsis thaliana* (Plakidou-Dymock *et al.*, 1998). The protein encoded by the GCR1 gene shows high amino acid similarity to 7-transmembrane receptors from three different families, and is involved in cytokinin signal transduction. The cloning and characterisation of IAR1, a gene required for auxin conjugate sensitivity in *Arabidopsis* has also been reported (Lasswell *et al.*, 2000). IAR1 structurally resembles and has detectable sequence similarity to a family of metal transporters, and was shown to encode a novel protein with seven predicted transmembrane domains, with homologues in *Drosophilla*, nematodes and mammals. G proteins have thus been implicated in a number of signalling events in plants.

Finally, a further potential application of this initial work is to use membrane targeting sequences for other bioactive proteins that could be expressed at the plant cell surface. As a scientific tool the immunoglobulin transmembrane sequence could be useful as a marker for analysis of protein expression in plants, as well as having applications in the sorting of protoplasts by fluorescence-activated cell sorting (FACS) [Galbraith, 1989]. In mammalian cells, cross linking of surface proteins often leads to capping and intemalisation and if this occurs in plants, cell surface antibodies could be used as a means of internalising foreign molecules into protoplasts.

CHAPTER 5:

RESULTS III

CONTENTS

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Expression and assembly of a secretory antibody in transgenic tobacco plants

The first studies on passive immunotherapy against dental caries were performed with Guy's 13, a murine IgG monoclonal antibody that binds to streptococcal antigen I/II, the cell surface adhesion protein of *Streptococcus mutans*. The oral cavity contains a complex oral flora and a number of bacterial proteases. For this reason, passive immunotherapy with a secretory antibody would be preferable to IgG. Indeed, the predominant form of immunoglobulin in mucosal secretions is secretory IgA, which, as described in the Literature Review, has higher functional affinity and is more resistant to proteolytic degradation than IgG.

An engineered form of the Guy's 13 gamma heavy chain had already been expressed in plants, in which the Cγ3 domain was replaced by the Cα2 and Cα3 domains of a murine alpha heavy chain (Ma *et al.*, 1994). It had also been demonstrated that this chimaeric heavy chain (designated γ/α heavy chain) assembled correctly with the original kappa light chain to form functional antibody (designated IgA/G).

The aims of this part of the study were to:

1) determine if higher assembly forms of immunoglobulin could be expressed in plants, initially by co-expression of Ig light, heavy and joining chains to produce dimeric antibodies in a single plant.
2) if successful, to co-express a further recombinant protein – secretory component – in order to assess the potential for assembly of a monoclonal secretory antibody.
3) examine the efficiency of assembly of Guy's 13 secretory antibody,
4) perform functional studies to compare the recombinant plant SIgA to the native murine IgG.

For these studies transgenic *Nicotiana tabacum* plant lines that had previously been generated were used (Ma *et al.*,1994). These expressed;

1) Guy's 13 MAb heavy chain (γ1)
2) Guy's 13 MAb light chain (κ)

3) Chimaeric Guy's 13 MAb heavy chain (γ1/α)

4) Chimaeric Guy's 13 MAb heavy chain (γ2/α)

5) Murine Joining (J) chain*

6) Rabbit secretory component (SC)

*The DNA and amino acid sequences are shown in the Appendix.

The parent singly transformed parent plants (κ, γ1, γ2/α and γ1/α) were initially screened for transgene expression by ELISA (data not shown). The crossing of light and heavy chain-secreting plants resulted in; 3 out of 10 F_1 progeny plants expressing kappa and gamma chains together, 4 of 17 plants expressing both kappa and the γ1/α heavy chain, and 3 of 8 plants expressing both kappa and the plant γ2/α heavy chain together, as demonstrated by Western blotting and ELISA (section 5.1 below).

5.1 Detection of recombinant immunoglobulin chains and assembled monomeric immunoglobulins in transgenic tobacco plants

The F1 progeny of the first cross between light and heavy chain transgenic plants was expected to include plants that co-expressed both chains and assembled them into functional antibody monomers. Western blots of representative plants from this cross are shown in Figures 5.1 and 5.2.

Figure 5.1 Panel A demonstrates the expression of Guy's 13 light chains under reducing conditions. Using the murine hybridoma culture supernatant as a positive control, Guy's 13 light chain is detected at approximate M_r 25K (lane a). No cross-reactive bands were detectable in wild type non-transformed plants (lane e). However in plants expected to co-express light chain with the Guy's 13 γ1, γ2/α and γ1/α heavy chains, light chain of the predicted M_r was detected (lanes b, c and d respectively). Figures 5.1B and 5.1C show the Guy's 13 heavy chains expressed in the same plants. Using an anti-murine γ1 heavy chain antiserum, the native murine Guy's 13 heavy chain from hybridoma culture supernatant is detectable as a double band under reducing conditions (Figure 5.1B, lane a). The M_r is approximately 55K, and the difference between the two bands is due to glycosylation differences. In plants, Guy's 13 γ1 heavy chain is detectable predominantly as a single band (lane b), which may indicate that all the heavy chains are glycosylated in the same way.

The heavy chains of plant γ1/α and γ2/α were detected with an anti-alpha heavy chain antiserum under reducing conditions (Figure 5.1C, lanes c and d). The heavy chain of plant γ1/α (lane d) has a relative molecular mass of approximately M_r 60K and the plant γ2/α heavy chain is larger (approximate M_r 70K). These findings are consistent with the molecular weights predicted by sequence analysis, as the γ2/α heavy chain includes an extra immunoglobulin domain in comparison with the γ1/α and the native γ1 heavy chains. A number of other bands were detected in the transgenic plant extracts. These are likely to represent proteolytic fragments of either light/heavy chain complexes, or of the heavy chain, as no bands were detected in the extract from the wild type plant (lane e).

The same samples were also run under non-reducing conditions to assess the assembly of immunoglobulin molecules (Figure 5.2). Detection was with a labeled anti-kappa light chain antiserum in all cases. All three transgenic plant lines assembled immunoglobulin of the expected M_r for their respective full-length antibody. The plant Guy's 13 antibody (lane b) has the same M_r as the mouse Guy's 13 (lane a) - approx. M_r 170K, but the plant γ2/α (lane c) and plant γ1/α (lane d) antibodies have slightly higher M_r as predicted from the differences in the heavy chain sequences. Again, several smaller proteolytic fragments were also detected, which is consistent with the fact that a number of proteases are released by plants during the antibody extraction procedure (Hiatt *et al.*, 1989). That these are antibody fragments is confirmed by the absence of any detectable bands in the wild type plant extract (lane e).

5.2 ELISA results

The Western blot results demonstrated co-expression of light and heavy chains in plants, and the assembly of higher molecular weight complexes which were of the expected M_r for immunoglobulin tetramers. In order to confirm the assembly of immunoglobulins, and their correct functionality, an antigen-specific ELISA was used to demonstrate the presence of functional antigen binding sites.

Titration curves for extracts from three representative transgenic plants binding to the specific streptococcal antigen (SA I/II) are shown in Figure 5.3. Specific antibody was detectable in all three transgenic plant extracts, and the titration curves were similar to that of the murine hybridoma cell culture supernatant used at the same concentration. The

binding of the G1/A plant antibody appeared to be slightly lower than the other antibodies, although the titration curve was similar. No SA I/II binding was detected in the negative control plant, nor did extracts from plants individually expressing light or heavy chains have binding activity. These findings demonstrate that the transgenic plants expressing both light and heavy chains assembled the antibody molecule correctly to form a functional antigen binding site and confirmed that single light or heavy chains are not capable of binding the antigen.

Based on these results, it was decided to use the γ2/α heavy chain construct expressing plants for further crossing to develop secretory antibody plants. The reasons for this are discussed in more detail in the Discussion section of this chapter. An antibody expressing plant homozygous for both light and γ2/α heavy chains was generated by a self-fertilisation, followed by screening of the resulting seeds by back-crossing with wild type plants. The parent plant was identified as homozygous for heavy and light chains if all the offspring tested (12 out of 12) bound to SA I/II antigen in the ELISA.

5.3 Identification of transgenic plants expressing J chain

Plants had previously been regenerated following transformation with the gene encoding murine J chain. These were initially screened by ELISA and Western blotting using a variety of antisera raised against murine and human J chain (kindly supplied by Dr. M. Parkhouse, and Nordic Pharmaceuticals), but without success.

To confirm the sequence of the J chain insert, the J chain construct inserted into transgenic plants was re-isolated from regenerated plants and cloned into the plasmid Bluescript KS^+. DNA sequencing identified a single amino acid change from the expected sequence – an arginine residue in place of a lysine at amino acid position number 130. This is a conservative change, which may simply be a mouse strain variation. The complete DNA sequences of murine J chain and plant J chain are shown in the Appendix.

Although expressed protein could not be detected in plant extracts, it was felt that this was due to the quality of the antisera available as well as low levels of expression. Putative J chain transgenic plants were subsequently screened using the reverse transcriptase-polymerase chain reaction (RT-PCR). Heterozygous plants selected on this basis were used for further cross fertilisation studies.

5.4 J chain RT-PCR

Figure 5.4A shows the RT-PCR products derived from reactions using J chain specific oligonucleotides (lanes a – d) with cDNA prepared from plant mRNA. The sequences of the J chain specific oligonucleotides are shown in the Appendix. A positive J chain control plasmid (described in the Appendix) is shown in lane a. A product of the expected size, 700 bp, was detected from cDNA derived from a plant transformed with murine J chain DNA (lane b). No PCR products were detected in reactions from either a wild type plant (lane c), or a monomeric Guy's 13 antibody expressing plant (lane d). Negative control reactions were performed with mRNA without any reverse transcriptase (lanes e –g), and no bands were detected, demonstrating the absence of any cDNA in the samples.

As a control experiment to confirm the isolation of mRNA, an RT-PCR was carried out with rubisco specific oligonucleotides (sequences of the rubisco oligonucleotides are shown in the Appendix), using cDNA prepared from plant mRNA. A product of approximately 600 bp, the expected size of the rubisco fragment was detected in each of the reactions performed (Figure 5.4B, lanes a – c). However when an RT-PCR was performed using the same mRNA in the absence of any reverse transcriptase, the 600 bp product was not detected (Figure 5.4B, lanes d –f). This result demonstrates the presence of RNA specific for the constitutive rubisco gene in the extracts of plant RNA, and acts as a control for the mRNA preparations.

The heterozygous plants that expressed the murine J chain were crossed with homozygous plants that expressed IgG/A (with $\gamma 2/\alpha$ heavy chain). Immunoblot screening of crude plant extracts from the progeny of this cross demonstrated that in 4 out of 16 plants, three major bands could be detected under non-reducing conditions with an anti-mouse kappa chain specific antiserum. An immunoblot of one representative plant extract is shown (Figure 5.5) alongside the parent plant extract expressing the monomeric IgG/A and Guy's 13 murine hybridoma culture supernatant. As before, murine Guy's 13 IgG has a slightly faster mobility than the chimaeric IgG/A molecule that has an approximate M_r 210-220K (lanes d and c). The same M_r 210-200K band was observed in the extract from the plant co-expressing light, $\gamma 2/\alpha$ heavy and J chains (lane b). In addition, a higher molecular weight band, approximately twice the weight of the monomeric IgG/A was observed which

is likely to represent dimerised antibody (dIgA/G). No cross-reactive bands were observed in extract from wild-type non-transformed plants.

5.5 Cross fertilisation of dimeric IgA/G and SC transgenic plants

Transgenic plants expressing rabbit secretory component were identified by Western blotting using a specific antiserum kindly supplied by Dr. James Casanova (results not shown). Homozygous SC transgenic plants were generated by self-fertilisation, followed by screening of the resulting seeds by back-crossing with wild type plants. The parent plant was identified as homozygous for heavy and light chains if all the offspring tested (12 out of 12) bound to SA I/II antigen in the ELISA

Mature plants that expressed dIgA/G were crossed with a homozygous plant that expressed SC. Using one plant representing each of the primary transgenics (J chain and secretory component) and their filial recombinants (IgG/A, dIgA/G and SIgA/G), the presence or absence of 3 of the relevant chains (heavy, light and SC) was detected by Western blotting (Figure 5.6). The plant samples were run under reducing conditions with β-mercaptoethanol. Heavy and light chains (of expected M_r) were detected in all antibody expressing plants – IgG1 control, IgG/A, dIgA/G and SIgA/G, but not in wild type, J or SC transgenic plants (Upper and Middle Panels). Secretory component was only detected in the SC and SIgA/G plants (Lower Panel). Only the SIgA/G plants were shown to co-express the heavy, light and SC chains. No cross-reactive bands were observed in the wild-type non-transformed control plant extract.

Under non-reducing conditions, increasingly higher molecular weight immunoreactive bands were detected as more of the polypeptide chains were co-expressed. Thus the SIgA/G plant that co-expressed light, heavy and J chains with secretory component produced a high molecular mass band of approximate M_r 470 (SIgA/G) in protein immunoblot analysis using an anti-mouse kappa chain specific antiserum (Figure 5.7, Panel a, lane 5). Such a molecular size is consistent with that expected for a secretory Ig and the relationship of this band with those seen previously in monomeric immunoglobulin and dimeric immunoglobulin expressing plants is illustrated in Figure 5.7 (Panel a, lanes 3 and 4). This immunoblot was probed with an anti-kappa light chain reagent, as the kappa chain is common to all forms of the Guy's 13 antibody expressed in plants.

Probing the same plant extracts with antiserum specific to SC confirmed that the high molecular mass protein contained SC (lane 7), whereas none of the other major Ig bands were recognised. In a transgenic plant that secreted SC alone without other immunoglobulin chains, no bands were detected with the anti-light chain antiserum (lane 6). Nor were any other high M_r proteins detected under non-reducing conditions with anti-SC antiserum (lane 9). Hence there was no evidence that SC assembled with endogenous plant proteins or produced multimers. No cross-reacting proteins were detected in extracts from the wild type control plant (lanes 2 and 10).

Complete dissociation of SC from Ig heavy chains only occurred under reducing conditions, suggesting that the SC chain is at least partially covalently linked in the fully assembled SIgA/G molecule. The molecular mass of the major SC band under reducing conditions was about M_r 50 kDa, slightly lower than expected (M_r 66.5 kDa), and slightly lower in SC transgenic plants as compared with SIgA/G plants (Figure 5.6). This may be due to proteolysis, which can occur in the intact plant or during sample preparation. SC bound to dimeric IgA is often found proteolysed to smaller but biologically active forms *in vivo* (Ahnen *et al.*, 1986). However in the protein immunoblot analysis under non-reducing conditions, the molecular mass difference between dIgA/G and SIgA/G was approximately M_r 70 kDa, as expected (Figure 5.7, lanes 4 and 5).

5.6 Correct assembly of SC to immunoglobulin

In mammals, the assembly of SC with antibody requires the presence of the J chain (Brandtzaeg & Prydz, 1984), we now investigated whether this was also the case in plants. Plants expressing monomeric IgG/A (without J chain) were cross-fertilised with homozygous SC-expressing plants. In the progeny that co-expressed IgG/A and SC, only the monomeric IgG/A form of the antibody was detected in Western blot, and no higher molecular mass forms (Figure 5.8 Upper Panel, lanes 1 and 2). Antiserum to SC recognised free SC but did not recognise proteins associated with Ig (Figure 5.8 Lower Panel). This result was confirmed in all plants examined.

Crossing a plant that expresses dimeric IgA/G with a homozygous plant that expresses SC is predicted to generate 8 possible genotypes, shown in Table 2. However only one of these is predicted to contain copies of the heavy, light, J chain and SC genes (Table 2, highlighted in red). Subsequently, 2 out of 15 plants tested contained a M_r 470 kDa

SIgA/G molecule (a representative plant is shown in lane 3). No cross-reactive bands were detected in the wild-type non-transformed plant (lane 4). This finding confirmed the requirement of the J chain for SC association with Ig and suggests that the nature of this association is the same in plants and mammals.

Genotypes from cross between dimeric IgA/G and SC plants
K J α/γ SC
K J SC
K α/γ SC
J α/γ SC
K SC
α/γ SC
J SC
SC

Table 2: Possible genotypes from crossing heterozygous dimeric IgA/G plants with homozygous SC plants.

5.7 Functional antibody studies of recombinant plant IgG and SIgA/G

Functional antibody studies were carried out with samples from transgenic plants expressing either; SIgA/G, dIgA/G, IgG/A, SC, or J chain using antigen-specific ELISAs. Detection was with either an anti-light (kappa) chain reagent or anti-SC antiserum. Guy's 13 hybridoma cell culture supernatant (IgG) was used as a positive control.

All plants expressing antibody light and heavy chains assembled functional antibodies that specifically recognised SA I/II (Figure 5.9 Panel A). The levels of binding and titration curves were similar to those of the original mouse hybridoma cell supernatant. No SA I/II

binding was detected with wild type plant extracts, or from plants expressing only the J chain or SC.

The binding of antibody to immobilised purified SA I/II was also detected with antiserum to SC (Figure 5.9 Panel B). In this assay, only the SIgA/G plant antibody binding was detected, and not the functional antibodies in either the IgG/A or the dIgA/G plants. These results confirm that SC was assembled with antibody in the SIgA/G plant but did not interfere with antigen recognition or binding.

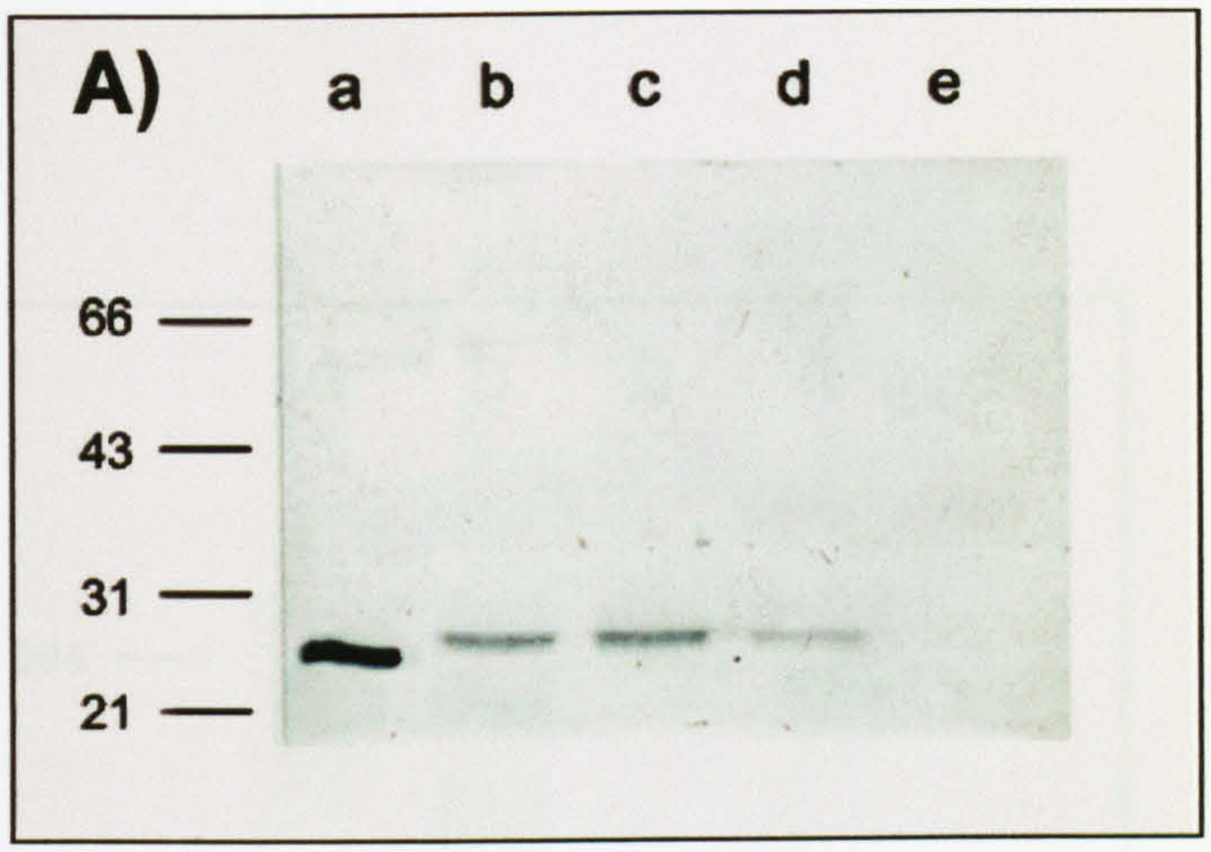
Binding of the plant secretory antibody to antigen in its native bacterial context was also confirmed (Figure 5.9 Panel C). In an ELISA using *S. mutans* whole cells as antigen, specific binding was observed in the extract from SIgA/G plants using the anti-secretory component antiserum. As with SA I/II, no binding was detected in any of the other transgenic plant extracts.

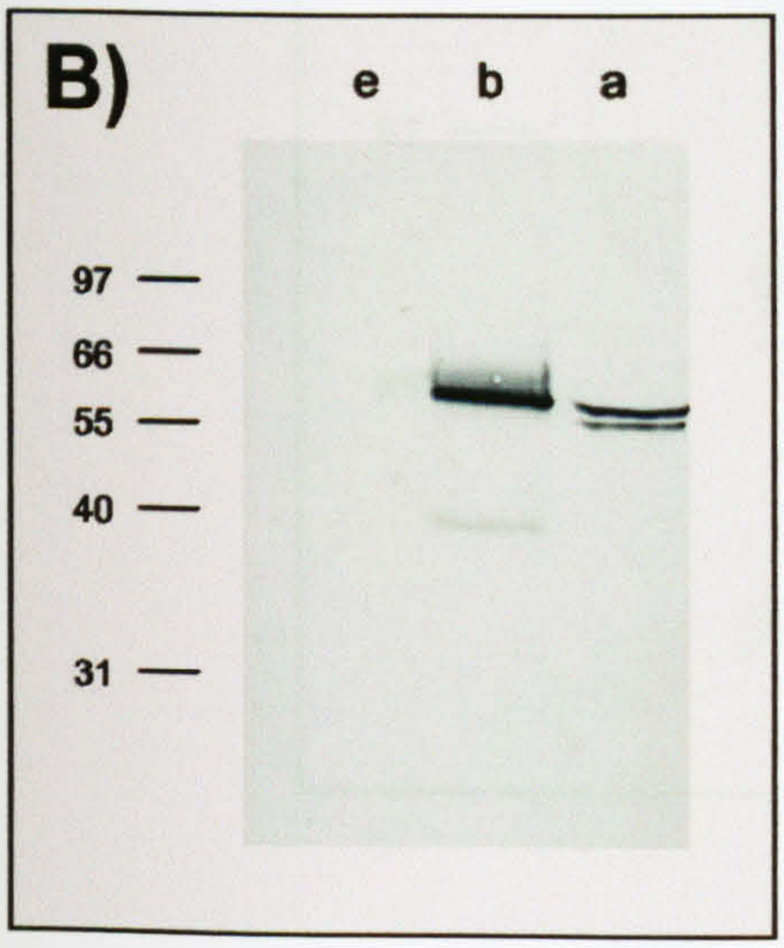
5.8 Levels of expression and efficiency of assembly of recombinant SIgA/G in plants

The assembly of functional IgG molecules in plants has been reported as being very efficient (Hiatt *et al.*, 1989), based on the expression levels observed. The expression levels of functional SIgA/G in tobacco leaves were measured in 12 different transgenic plants, by an antigen specific ELISA using Guy's 13 IgG at known concentration as the standard. The data are shown in Figure 5.10. The expression levels are expressed as a percentage of the total soluble protein (TSP) content of each plant extract, (as measured by the Bradford protein assay). No detectable antibody binding was observed in WT non-transformed plants. Two IgG plants expressed IgG at approximately 1 % of TSP when using anti-kappa chain antiserum for detection, but no binding activity was detected using the anti-secretory component antiserum in these plants. In the SIgA/G plants, using the anti-secretory component antiserum for detection, a range of levels were observed, with an average of 5.2 % and the highest expression level representing 8 % of TSP.

Densitometric analysis (Syngene GeneGenius™ image analysis software, Synoptics Ltd., Cambridge) of the Western blots suggests that in the dIgA/G, the dimeric antibody population constitutes a major proportion - almost 57 % - of the total antibody (Figure 5.5, lane 1). In the secretory IgA/G plants, the SIgA/G complex represents up to approximately 40 % of the total assembled antibody (Figure 5.8 lane 3). However protein immunoblot

analysis potentially underestimates the total extent of assembly of SIgA/G because it only detects antibody that is covalently linked to SC, whereas SIgA can occur *in vivo* as a mixture of covalently and non-covalently linked molecules (Schneiderman *et al.*, 1989; Knight *et al.*, 1975).





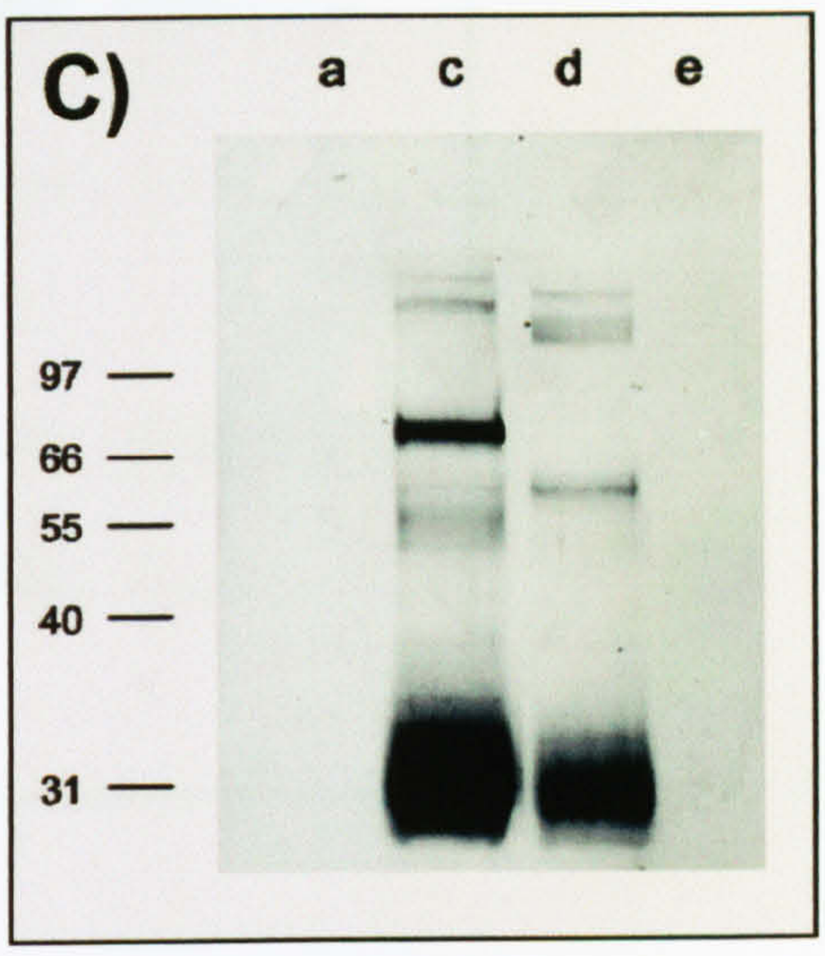


Figure 5.1: Western blot analysis of plant samples reduced with 5 % (v/v) β-mercaptoethanol. Samples were separated by 10 % SDS-PAGE. Detection was with; goat anti-mouse κ chain antiserum (Panel A); goat anti-mouse γ1 chain antiserum (Panel B); goat anti-mouse α chain antiserum (Panel C). Samples are; a – Guy's 13 mouse hybridoma culture supernatant, b – plant Guy's 13, c – plant γ2/α, d – plant γ1/α, e – wild type non-transformed plant. Relative molecular masses (x 10^3) are indicated.

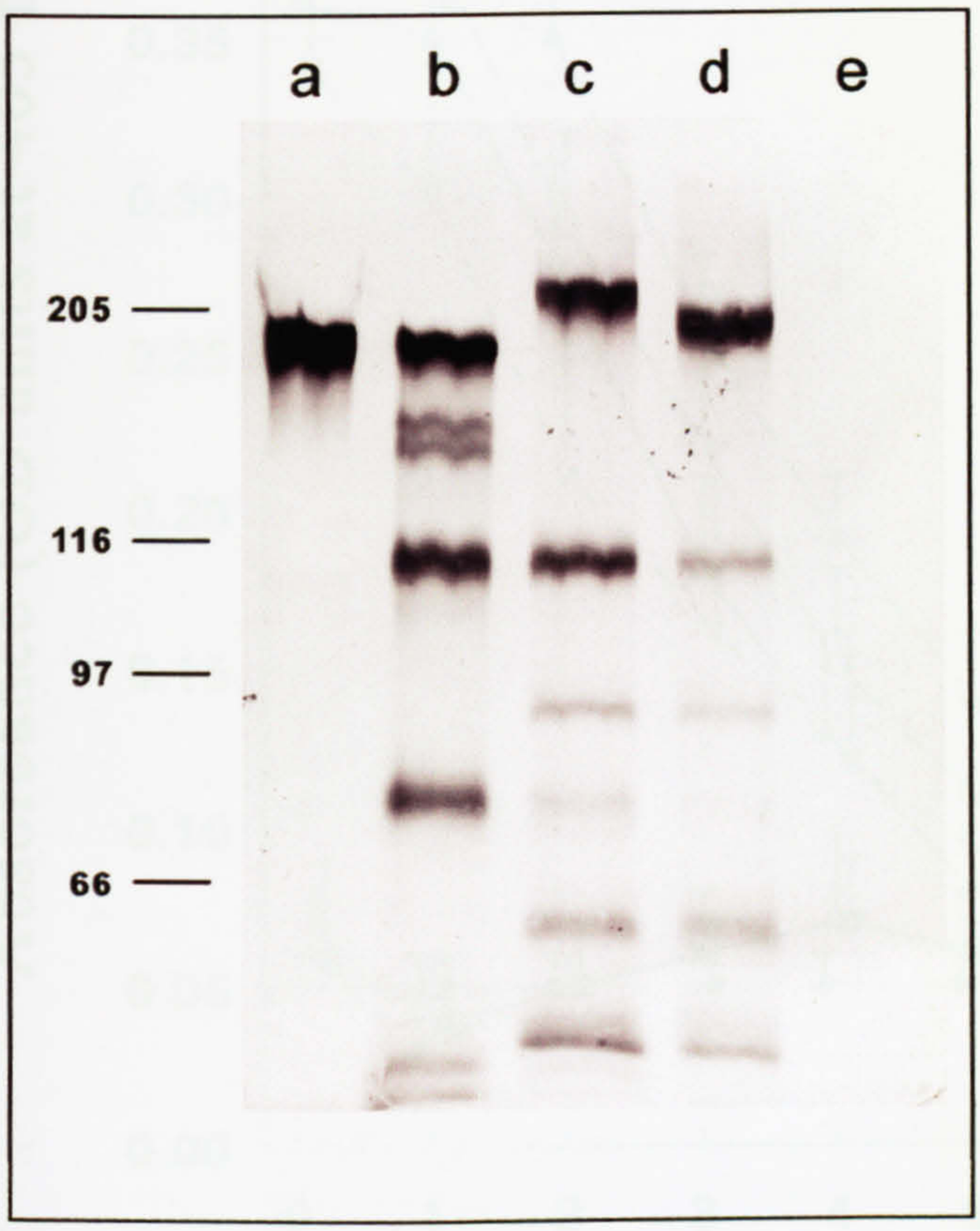


Figure 5.2: Western blot analysis of plant samples separated by 6 % SDS-PAGE under non-reducing conditions. Detection was with goat anti-mouse κ chain antiserum, followed by alkaline phosphatase-labeled rabbit anti-goat IgG antiserum. Samples are; a – Mouse Guy's 13 hybridoma culture supernatant, b – plant Guy's 13, c – plant γ2/α, d – plant γ1/α, e – wild type non-transformed plant. Relative molecular masses ($x10^3$) are indicated.

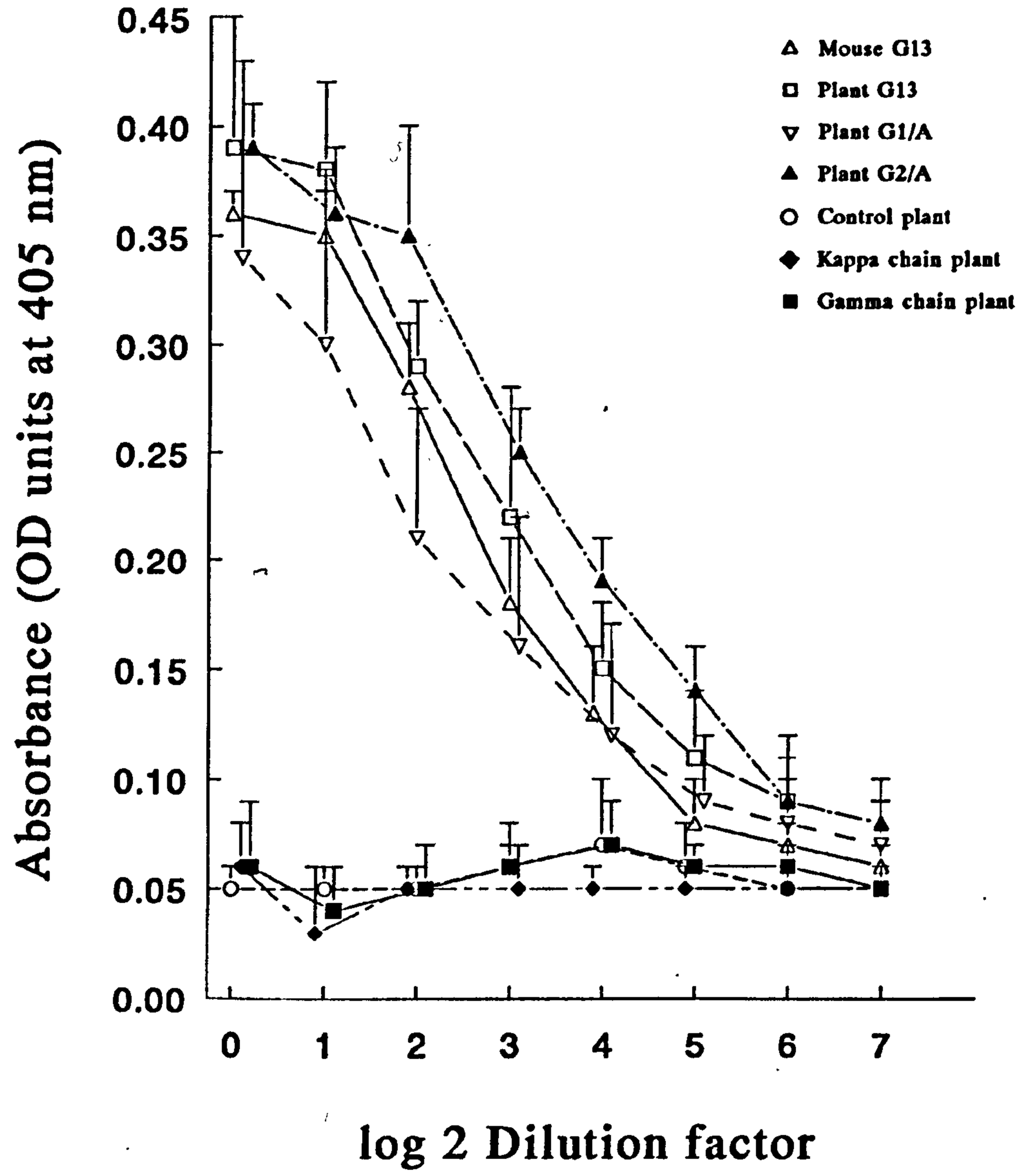


Figure 5.3: Binding of plant Guy's 13 samples to purified SA I/II in ELISA, detected using a horseradish peroxidase-conjugated goat anti-mouse light and heavy chain antiserum. The results are expressed as the mean absorbance +/- standard deviation of duplicate results from three separate experiments. The starting concentration of each antibody sample was adjusted to 1.5 μg/ml, and used in serial twofold dilutions.

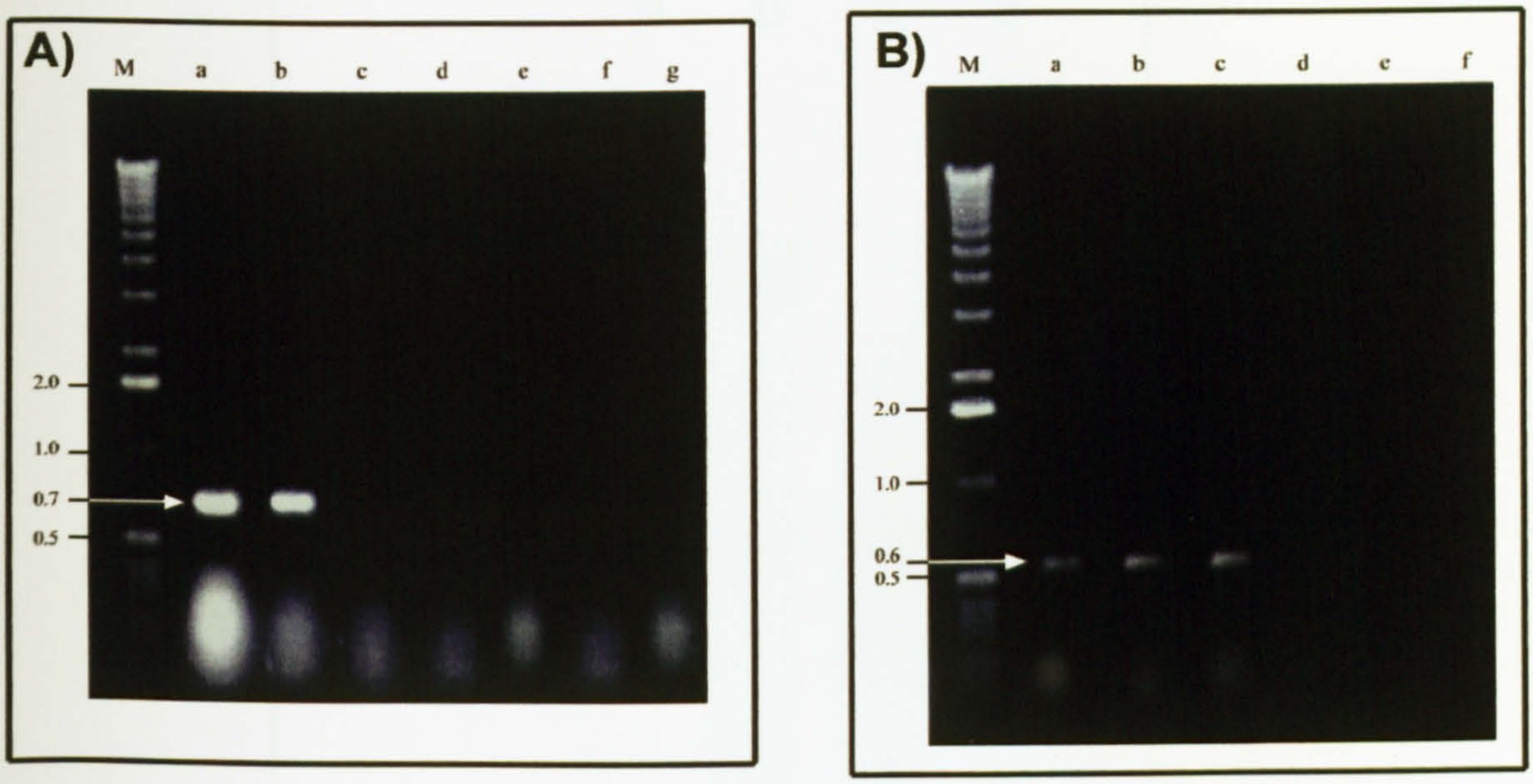


Figure 5.4: Agarose gel electrophoresis of reverse transcriptase polymerase chain reactions (RT-PCR) performed using samples of plant mRNA. Reaction products were visualised with ethidium bromide staining under UV light.

A) PCR was performed with J chain specific oligonucleotides (Appendix). Samples are; a – positive J chain control plasmid; b – transgenic plant expressing the J chain; c – wild type plant; d – transgenic plant expressing monomeric Guy's 13 IgG. Negative control reactions are shown in lanes e – g, in which plant mRNA was used in the absence of any reverse transcriptase; e – transgenic plant expressing the J chain, f– wild type plant, g- transgenic plant expressing monomeric Guy's 13 IgG.

B) PCR was performed with rubisco specific oligonucleotides. (Appendix). Samples are; a – wild type plant, b – transgenic plant expressing the J chain, c – transgenic plant expressing monomeric Guy's 13 IgG. Negative control reactions are shown in lanes d – f, in which plant mRNA was used in the absence of any reverse transcriptase; d – wild type plant, e - transgenic plant expressing the J chain, f – transgenic plant expressing monomeric Guy's 13 IgG.

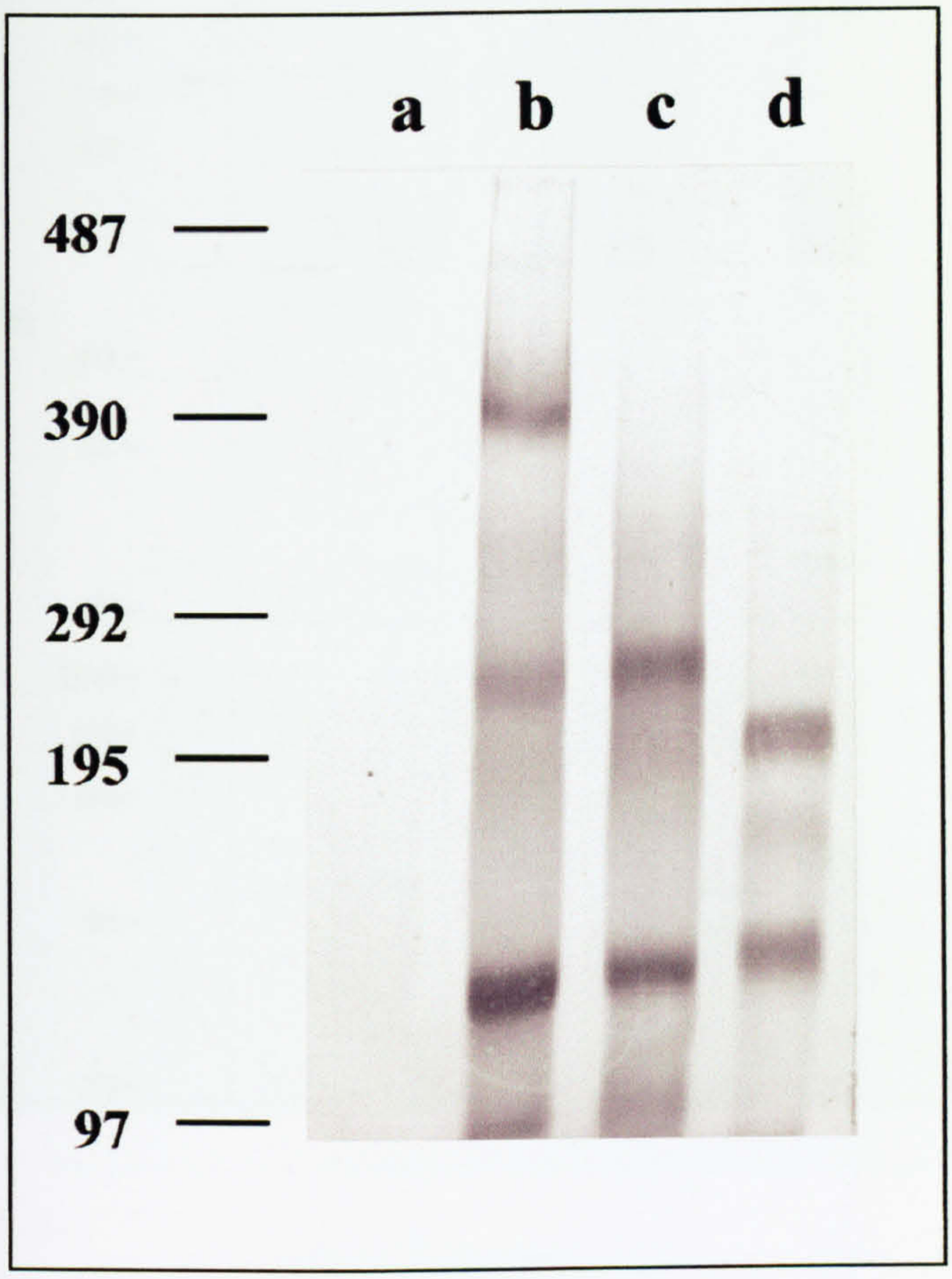


Figure 5.5: Protein immunoblot of plant extracts prepared under non-reducing conditions, detected with antisera to the mouse κ light chain. Samples were prepared and separated on 4 % (w/v) SDS-PAGE. The samples are; a – wild type plant, b - transgenic plant expressing modified heavy and light chain genes of Guy's 13 and the J chain, c – transgenic plant expressing modified heavy and light chain genes of Guy's 13, d – Mouse Guy's 13 hybridoma culture supernatant. Relative molecular masses ($x10^3$) are indicated.

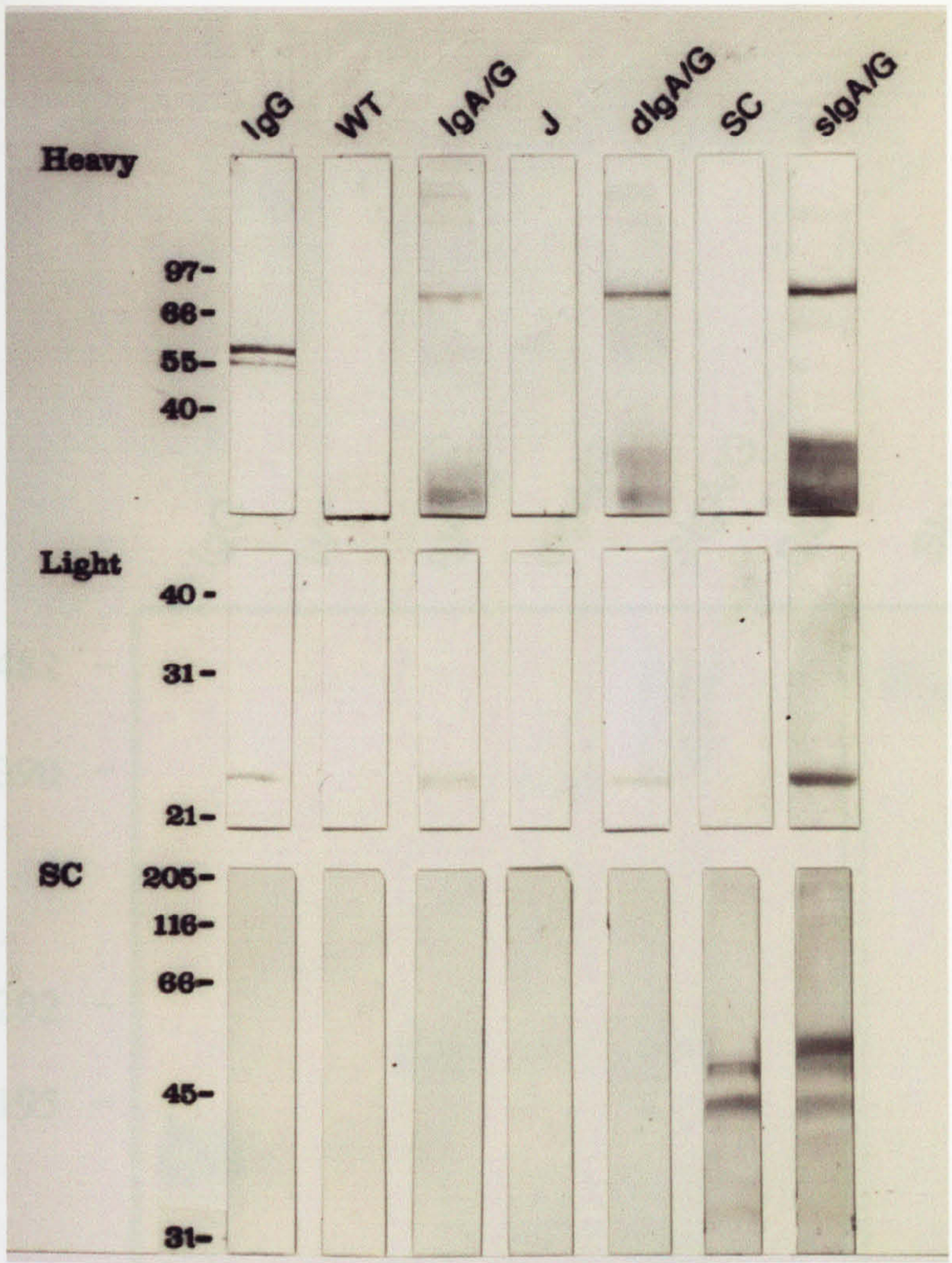


Figure 5.6: Protein immunoblot of plant extracts. Samples were separated on 10 % (w/v) SDS-PAGE under reducing conditions with 5 % (v/v) β-mercaptoethanol. Detection was with antisera to mouse γ1 heavy chain (upper panel), the mouse κ light chain (middle panel), or rabbit SC (lower panel).
Samples are; IgG – Guy's 13 hybridoma cell culture supernatant; WT – non-transformed wild type plant; IgG/A, transgenic plant expressing modified heavy and light chain genes of Guy's 13; dIgA/G, transgenic plant expressing modified heavy and light chain genes of Guy's 13 and the J chain; SIgA/G, transgenic plant expressing modified heavy and light chain genes of Guy's 13, the J chain, and SC; SC, transgenic plant expressing SC; and J, transgenic plant expressing the J chain. Relative molecular masses ($x10^3$) are indicated.

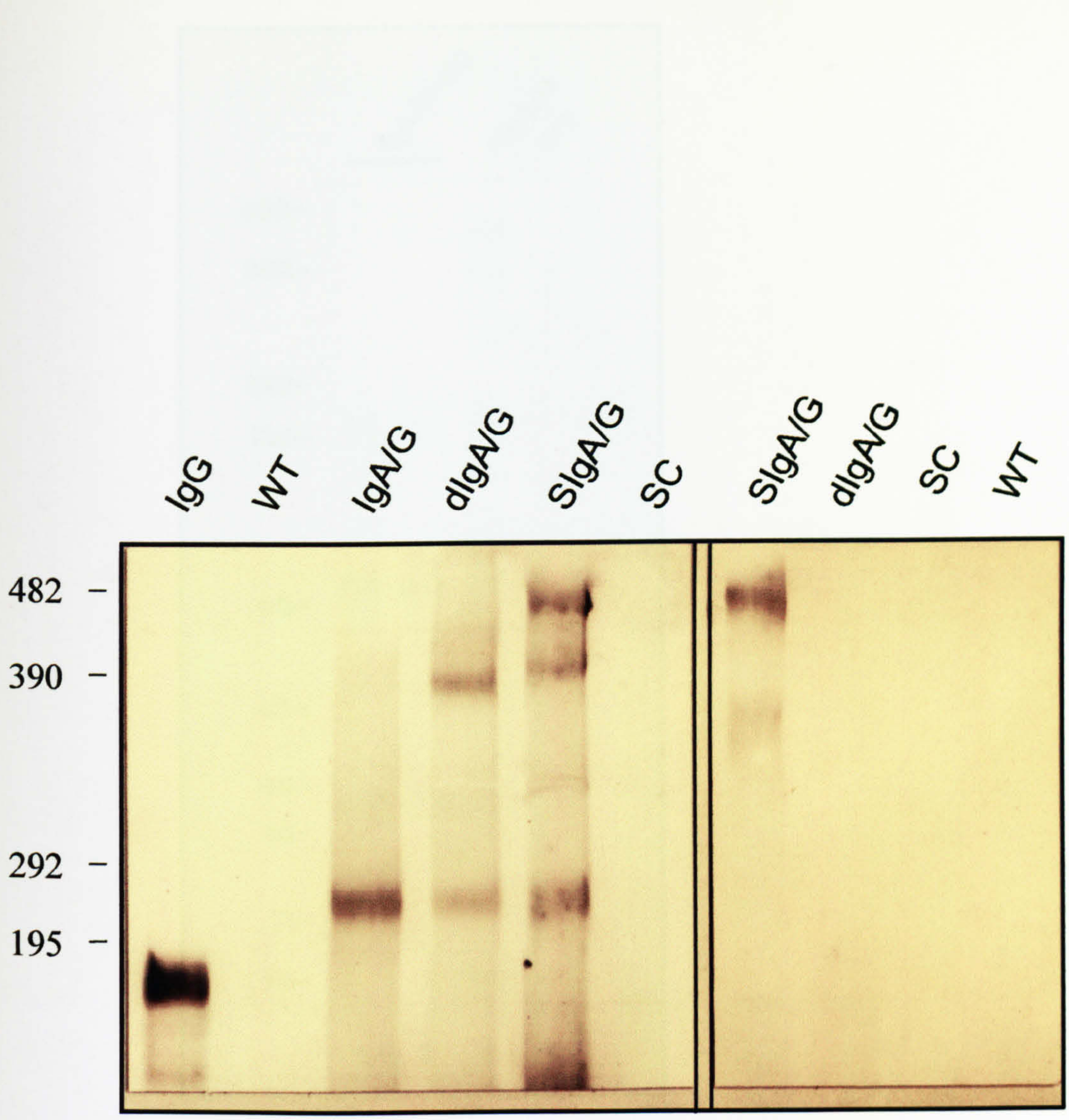


Figure 5.7: Protein immunoblot of plant extracts prepared under non-reducing conditions, detected with antisera to the mouse κ light chain (left lanes) or to rabbit SC (right lanes). Samples were prepared and separated on 4 % (w/v) SDS-PAGE. Samples are; IgG– Guy's 13 hybridoma cell culture supernatant; WT – non-transformed wild type plant; IgG/A, transgenic plant expressing modified heavy and light chain genes of Guy's 13; dIgA /G, transgenic plant expressing modified heavy and light chain genes of Guy's 13 and the J chain; SIgA/G, transgenic plant expressing modified heavy and light chain genes of Guy's 13, the J chain, and SC; SC, transgenic plant expressing SC; and J, transgenic plant expressing the J chain. Relative molecular masses ($x10^3$) are indicated.

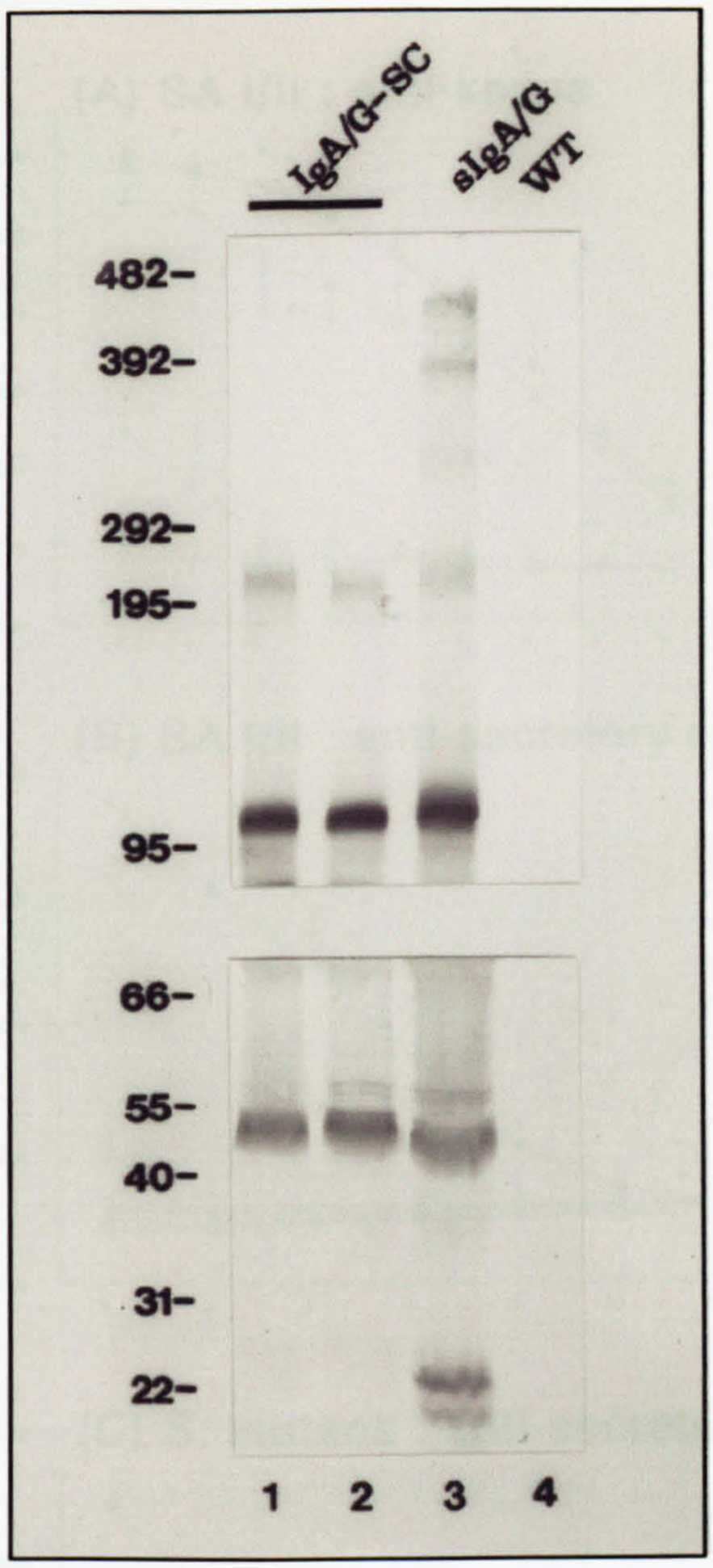


Figure 5.8: Co-expression of IgG/A with SC. Protein immunoblot of plant extracts.
Upper panel: Samples were separated on 4 % (w/v) SDS-PAGE under non-reducing conditions. Detection was with goat antisera to the κ light chain
Lower panel: Samples were separated on 10 % (w/v) SDS-PAGE under reducing conditions. Detection was with sheep antisera to SC.
IgA/G-SC lanes 1 and 2 are transgenic plants expressing modified heavy and light chain genes of Guy's 13 and SC; sIgA/G is a transgenic plants expressing modified heavy and light chain genes of Guy's 13, SC and J chain; WT is a non-transgenic wild type control plant. Relative molecular masses (x 10^3) are indicated.

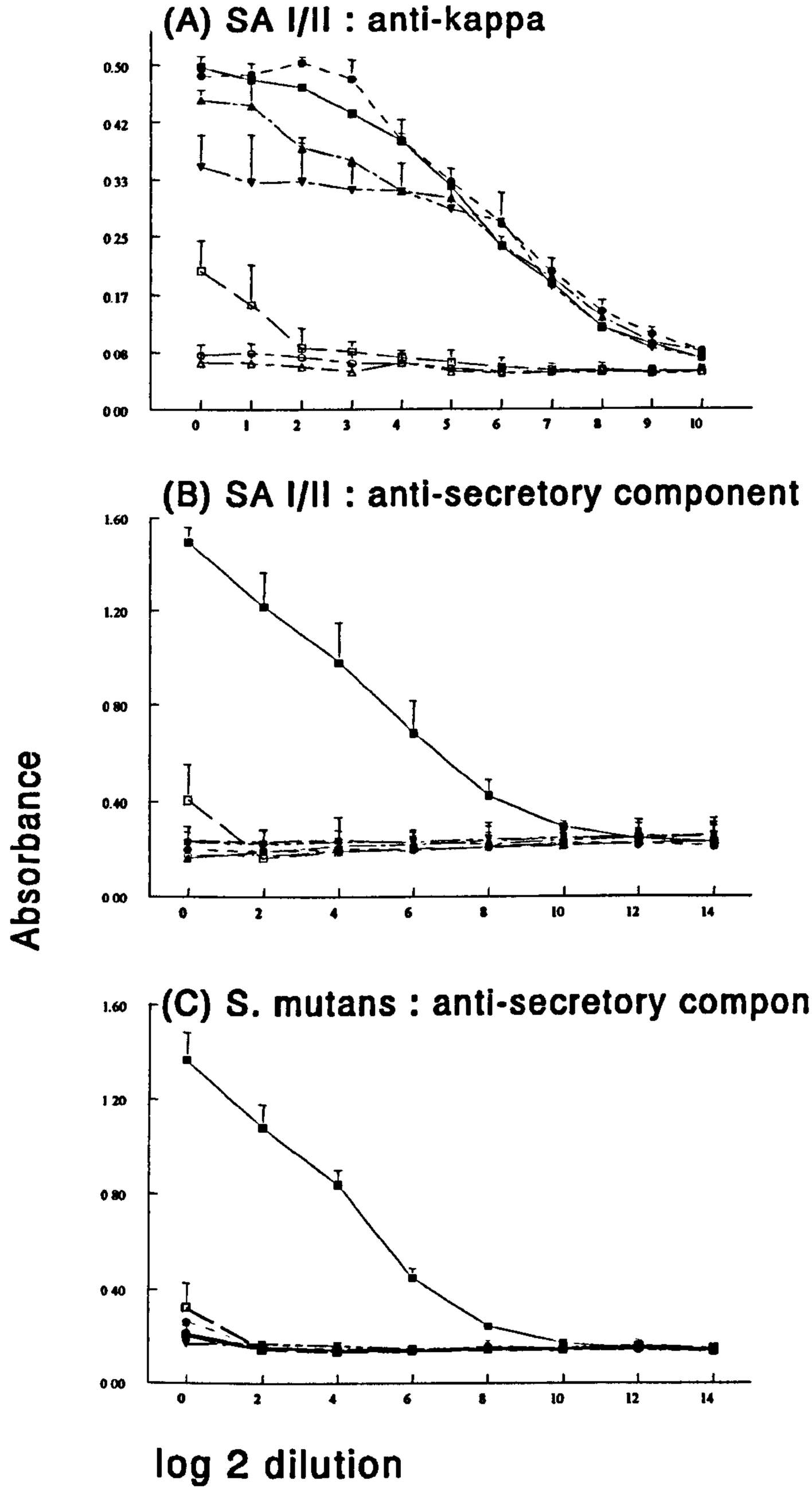


Figure 5.9: Functional antibody expression in transgenic *N. tabacum*. (A) Plant extract binding to purified SA I/II, detected with HRP-labeled antiserum to the κ light chain. (B) Plant extract binding to purified SA I/II, detected with sheep antiserum to SC followed by alkaline phosphatase-labeled donkey antiserum to sheep Ig. (C) Plant extract binding to streptococcal cells, detected with sheep antiserum to SC followed by alkaline phosphatase-labeled donkey antiserum to sheep Ig. Guy's 13 hybridoma cell culture supernatant (IgG) was used as a positive control. The initial concentration of each antibody solution was 5μg/ml. Dilution numbers represent serial double dilutions. Results are mean +/- standard deviation of three separate triplicate experiments. ■, SIgA/G; ●, dIgA/G; ▲, IgA/G; □, SC; O, J chain; Δ, wild type; and ▼, Guy's 13 hybridoma culture supernatant.

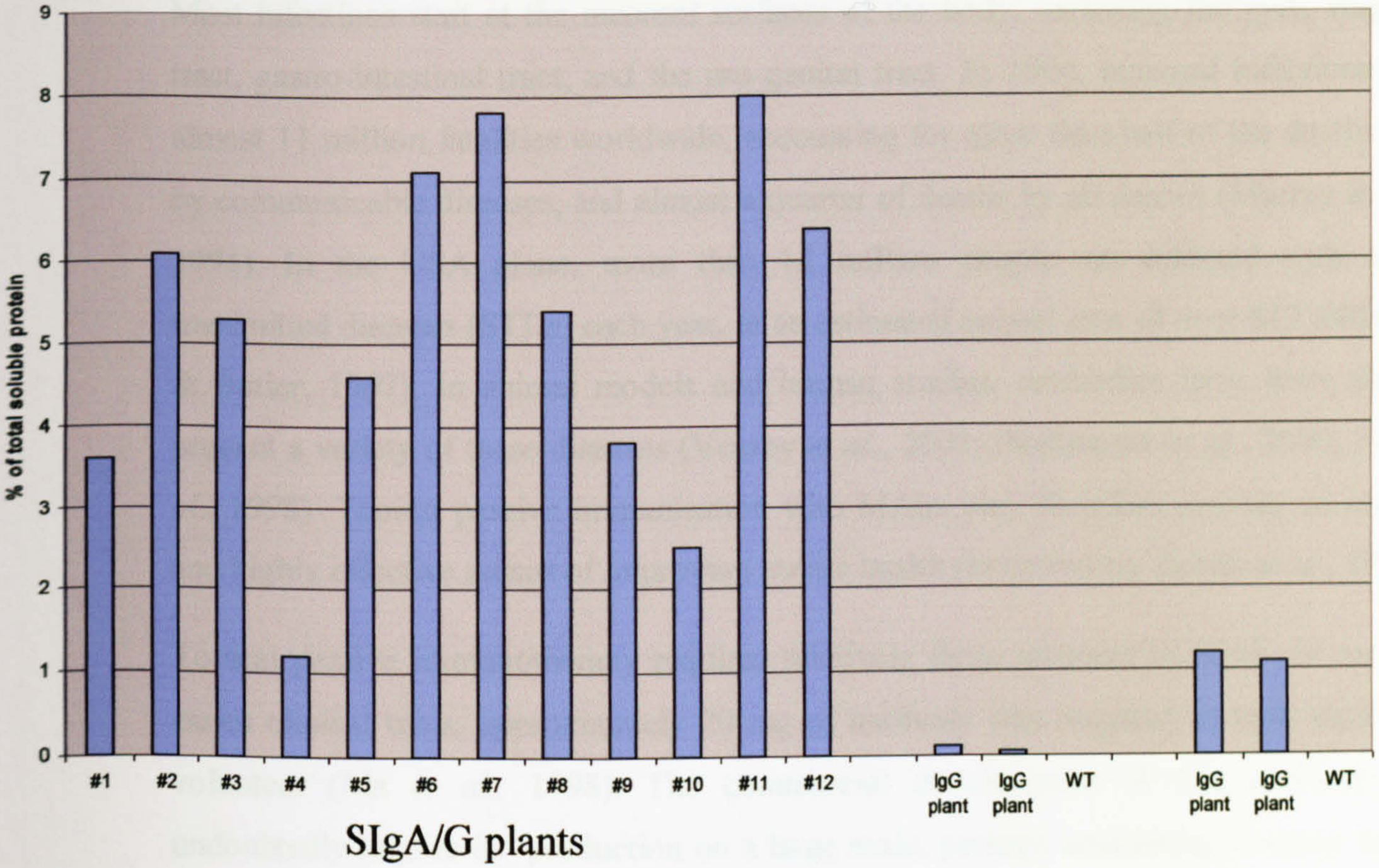


Figure 5.10: Antibody expression levels in transgenic plants. Antigen specific ELISA was used to determine the titration points for all samples. Plant extracts were detected with anti-secretory component antibody or anti-kappa chain antibody as indicated. Concentration values were determined by comparison with a Guy's 13 IgG standard. Values are expressed as percentage of total soluble protein.

5.9 Discussion

Most infections start at the mucosal surfaces of the body, including the eyes, respiratory tract, gastro-intestinal tract, and the uro-genital tract. In 1994, mucosal infections caused almost 11 million fatalities worldwide, accounting for more than half of the deaths caused by communicable diseases, and almost a quarter of deaths by all causes (Murray & Lopez, 1994). In the USA alone, more than 12 million people are infected with sexually transmitted diseases (STDs) each year, at an estimated annual cost of over $12 billion (Eng & Butler, 1997). In animal models and human studies, antibodies have been shown to prevent a variety of these diseases (Veazey *et al.*, 2003; Piedimonte *et al.*, 2000; Zeitlin *et al.*, 1998). Topical passive immunisation with MAbs may therefore provide an economic and highly effective means of improving public health (reviewed by Zeitlin *et al.*, 1999).

Topical passive immunotherapy requires relatively large amounts of MAb. In our dental caries clinical trials, approximately 20 mg of antibody was required to treat each human volunteer (Ma *et al.*, 1998). The commercial development of this antibody would undoubtedly require its' production on a large scale, perhaps amounting to many hundreds of kilogrammes of antibody per year in the UK alone, well beyond the capability of traditional technologies. For this reason, we have studied plants as an expression system for recombinant antibodies.

The most appropriate antibody isotype for mucosal delivery on most human mucosal surfaces is likely to be secretory IgA (SIgA). In the case of dental caries, a monoclonal SIgA antibody may possess several advantages over IgG in the prevention of *S. mutans* colonisation. One of the advantages to using secretory antibodies at mucosal surfaces is the resistance to proteolysis that has been observed with SIgA antibodies (Corthesy, 1997). It has been suggested that secretory antibodies might survive longer than serum antibodies in the mucosal environment due to the protective presence of the secretory component (Underdown & Dorrington, 1974; Mestecky & McGhee, 1987). Although the mouth does not contain significant amounts of indigenous proteases compared to the gastro-intestinal tract, it does contain a variety of bacterial proteases from the normal commensal oral flora. In our studies, the susceptibility of plant derived SIgA/G to pepsin digestion has been studied, and we have demonstrated a measurable increase in resistance to proteolytic degradation. This may turn out to be highly significant, as it suggests that oral delivery of

plant derived secretory antibodies for immunotherapy of gastric and duodenal infections could be possible. In further studies that were performed with the plant derived secretory IgA/G antibody, a significant *in vivo* benefit was demonstrated for the secretory antibody over IgG in terms of stability and survival in the oral cavity (Ma *et al.*, 1998). This was investigated by examining the survival of the two forms of Guy's 13 antibodies applied topically into the oral cavity. Six human volunteers were used and the baseline limits of detection were determined using control subjects who were given bovine serum albumin in PBS by the same method as that used for the MAbs. Following the application of either IgG MAb or SIgA/G solution to the teeth, both antibodies were detectable after 30 minutes. In subjects who received IgG, the recoverable antibodies on teeth and in saliva then decreased rapidly and were undetectable after 24 hours. In contrast, SIgA/G was detectable for at least 72 hours on teeth and for up to 48 hours in saliva. The detection assay required functional antigen binding and the SIgA/G antibody could be detected with both an anti-light chain as well as an anti-SC reagent which does not cross-react with either heavy or light chains. The results therefore suggest that the antibody recovered remained functionally intact in the oral cavity.

Another advantage of using plant derived secretory antibodies derives from the increased valency of secretory immunoglobulins as compared with IgG. It was previously shown that Guy's 13 IgG and the $F(ab')_2$ fragment were protective in humans, but that the Fab' fragment was not (Ma *et al.*, 1990). This suggested a requirement for at least bivalent antigen binding. The rationale for the use of the plant SIgA/G antibody was that there is a progressive increase in valency and avidity from the ineffective monomeric Fab', to the protective dimeric $F(ab')_2$ and IgG, to the tetravalent SIgA/G, which may correlate with protective efficacy. The increased avidity of secretory antibodies would augment bacterial aggregation and immune exclusion. Using the plant derived SIgA/G molecule, we have been able to demonstrate that the affinity of antigen binding is equivalent to that of the native IgG, however the avidity of binding was increased, as predicted by the dimeric form of SIgA/G (Ma *et al.*, 1998).

The binding affinity of the plant SIgA/G to streptococcal antigen I/II was compared with that of the native mouse IgG Guy's 13 by surface plasmon resonance (Ma *et al.*, 1998). The binding curves for both forms of antibody at equimolar concentrations were similar for both the association and dissociation phases. The values for k_a, k_d and Kd were calculated from

sensorgrams using five concentrations for each antibody. There was no difference between the deduced dissociation constants for plant SIgA/G (Kd = 5.2 x 10^{-10}M) and IgG (Kd = 1.3 x 10^{-9} M) monoclonal antibodies. This assay was optimised for a 1:1 molecular interaction, so it is unlikely that differences in avidity would be apparent. However like the ELISA results reported earlier in this chapter, surface plasmon resonance analysis does serve to confirm the correct assembly of the antigen binding site of the antibody in plants and shows that antigen recognition is unaffected by any differences in antibody structure, due to the complex nature of the SIgA/G molecule or variations between plant and murine glycosylation.

A comparison of the functional affinities of the two antibody preparations was determined using a competition ELISA. In this assay, addition of excess antigen in the fluid phase favours multivalent interactions with antibody (Ma *et al.*, 1998). Approximately 4-fold higher antigen concentration was required to inhibit binding of IgG to SA I/II by 50 % (IC_{50} = ~ 43 nM), compared with the plant SIgA/G (IC_{50} = ~ 11 nM), which demonstrates that the SIgA/G preparation had a higher functional affinity than IgG. As the dissociation constants of the two antibodies are the same (as shown by surface plasmon resonance), this difference can be accounted for by the higher avidity of the tetravalent SIgA/G and dIgA/G molecules compared with the bivalent IgG molecule (Steward & Chargelegue, 1997).

The most valid functional assay for this plant derived antibody was a human clinical trial, testing the protective efficacy against *Streptococcus mutans* colonisation in the oral cavity (Ma *et al.*, 1998). The plants prepared in this study were used to generate sufficient biomass for purification of 100 mg monoclonal secretory antibody by Planet Biotechnology Inc. This material was then used in a clinical trial.

The clinical protective efficacy of MAb Guy's 13 IgG had previously been established by preventing colonisation by *S. mutans* and dental caries in animals (Lehner *et al.*, 1985), as well as by preventing both exogenous colonisation (Ma *et al.*, 1989) and recolonisation by indigenous *S. mutans* (Ma *et al.*, 1990) in humans. The ability of the plant Guy's 13 SIgA/G preparation to prevent recolonisation by *S. mutans* was therefore determined alongside the murine IgG1 MAb (Ma *et al.*, 1998). Two control groups were included; one that was sham-immunised with saline and another that received a non-specific plant/bovine IgG preparation. The latter control was used to exclude the possibility that a non-specific

plant component might co-purify with immunoglobulins and prevent bacterial colonisation.

Volunteers were chosen who already harboured oral *S. mutans*. They were treated with topical chlorhexidine gluconate for nine days to deplete the oral flora and eliminate *S. mutans*. Thereafter, antibody or control solutions were applied directly to their teeth for three weeks, two applications per week. Samples of dental plaque and saliva were collected at intervals up to four months to monitor the recolonisation *of S. mutans*. In every subject, topical chlorhexidine reduced the levels of *S. mutans* to below detectable limits in both plaque and saliva. In saliva, recolonising *S. mutans* was initially observed after 21 days in one individual in the group receiving plant/bovine IgG. At 58 days, recolonisation of *S. mutans* was found in all subjects in both control groups, and levels continued to rise until the end of the experiment at 120 days. In contrast, neither the four subjects treated with the plant SIgA/G preparation nor the four treated with murine Guy's 13 IgG recolonised for the duration of the experiment. Similar results were found in dental plaque samples.

The specific nature of this immunotherapeutic approach was tested by monitoring the levels of Actinomyces, a Gram positive commensal bacterial species commonly found in the oral cavity, in subjects receiving either plant/bovine IgG or the plant SIgA/G preparation. As with *S. mutans*, Actinomyces spp. fell to undetectable levels following treatment with chlorhexidine, but in contrast to the finding with *S. mutans*, both the non-immunised and immunised groups recolonised within 21 days and the Actinomyces spp. were restored to pre-experimental levels by 88 days. This finding supports the specific nature of the action of Guy's 13 MAb, as application of the antibody had no effect on the recolonisation of Actinomyces spp. or the rate at which this bacterial species was re-established in the oral cavity.

The more widespread use of secretory IgA in passive immunisation has been hindered until now by the complexity of the molecule and technical difficulties in producing sufficiently large quantities. Previously both IgA and SIgA have been partially purified on a small scale from milk (Brandtzaeg, 1970), bile (Taylor & Dimmock, 1985), and transfected mouse myelomas (Terskikh *et al.*, 1994). One of the earliest methods involved the *in vitro* conjugation of SC with dimeric IgA from several different mammalian species (Mach, 1970). Free SC purified from both human and bovine milk was labeled with [^{125}I] and incubated with purified human myeloma polymeric IgA. Chromatographic analysis showed

that almost 90 % of the labeled free SC could be bound to the polymeric IgA (Mach, 1970).

Other attempts to generate SIgA involved the *in vivo* insertion of subcutaneous "backpack" tumours of hybridoma cells secreting monoclonal IgA against *Vibrio cholerae* (Winner *et al.*, 1991). IgA hybridomas that produced dimeric monoclonal IgA antibodies were generated from Peyer's patch lymphocytes after oral immunisation of mice with a strain of *Vibrio cholerae.* Hybridoma cells were then injected subcutaneously into 1 day old syngeneic mice, which resulted in the secretion of monoclonal SIgA onto mucosal surfaces (Winner *et al.*, 1991). This system was used as a novel experimental model to assess the protective actions of individual IgA antibodies, and demonstrated that a single monoclonal SIgA could protect mice against intestinal disease caused by oral challenge with a lethal dose of *V. cholerae.*

The co-expression of recombinant IgA with J chain using the baculovirus insect cell system has been described elsewhere (refer to the Literature Review), but unlike our experience with plants only a small proportion of the expressed antibody was dimerised, most remaining in a monomeric form (Carayannopoulos *et al.*, 1994).

Recombinant SIgA has been generated in an *in vitro* coculture system that simulates the *in vivo* receptor-mediated transport of dimeric IgA antibodies (Hirt *et al.*, 1993). Hybridoma cells that provide a continuous supply of dimeric IgA (dIgA) were co-cultivated with a monolayer of Madin-Darby canine kidney (MDCK) cells expressing rabbit polymeric immunoglobulin receptor (pIgR). Approximately 20 % of the dIgA transported into the apical medium of this coculture system was covalently linked to SC and recovered as SC-dIgA complexes (Hirt *et al.*, 1993). Doubling the number of hybridoma cells in the basolateral matrix doubled the concentration of IgA in the basal medium, but did not increase the rate of dIgA transport. The expression of pIgR was shown to be rate-limiting in the transport process.

Further efforts to produce recombinant SIgA focused on the *in vitro* combination of purified dimeric IgA and SC using recombinant baculo- and vaccinia viruses (Rindisbacher *et al.*, 1995) are described in the Literature Review. In another study, the hybridoma cell line ZACC3 expresses *V. cholerae* lipopolysaccharide (LPS)-specific mouse IgA molecules as a population of monomeric, dimeric and polymeric forms (Lullau *et al.*, 1996). A combination of ultrafiltration, ion-exchange chromatography and size exclusion

chromatography was used to separate out milligram quantities of these three forms. Dimeric IgA and polymeric IgA (pIgA) bound strongly to LPS, whereas the monomeric form of IgA form did not. SIgA was reconstituted *in vitro* by combining recombinant SC and purified dIgA and pIgA. Surface plasmon resonance-based binding experiments demonstrated that purified reconstituted SIgA and IgA molecules recognised LPS with the same affinity (Lullau *et al.*, 1996).

The first report of the production of SIgA by a single mammalian cell has been made using a murine polymeric IgA-producing myeloma cell secreting mouse-human chimaeric IgA1 specific for the hapten dansyl, transfected with cDNA encoding human SC (Chintalacharuvu & Morrison, 1997). Almost all of the expressed SC was secreted covalently associated in SIgA, and only a minor band of free SC was seen in the supernatant. To determine their *in vivo* stability, dimeric IgA and SIgA proteins were purified and introduced into the stomach of BALB/c mice for 150 minutes. Dimeric IgA (dIgA) was more rapidly eliminated from the mice than SIgA, and more total and antigen-specific SIgA was recovered from intestinal washes, suggesting that SIgA was more stable in the intestines than dimeric IgA. However SDS-PAGE analysis of the IgA precipitated with antigen showed a major band corresponding to Fab' fragments in intestinal washes from mice given either SIgA or dIgA. Immunoprecipitates of anti-α and anti-κ chain also showed a major band corresponding to Fab' and Fc fragments and several minor higher molecular mass bands (Chintalacharuvu & Morrison, 1997).

A correctly assembled chimaeric mouse-human SIgA specific for the respiratory syncytial virus F glycoprotein has been expressed recently in CHO cells (Berdoz *et al.*, 1999). An advantage to using CHO cells is that they secrete proteins with N- and O-linked glycosylation patterns similar to those found in human cells, and furthermore CHO cells can also be grown to high density in bioreactors (Parekh *et al.*, 1989). A single CHO cell was sequentially transfected with expression vectors carrying respectively chimaeric heavy and light chains, human J chain, and human SC, with independent selective markers (Berdoz *et al.*, 1999). In the best producing clones, up to 20 μg of recombinant SIgA was recovered per 10^6 cells in 24 hours. Although this technique may appear to be capable of producing sufficient quantities of SIgA for immunotherapy, gene amplification might not favour higher expression levels, as mammalian cell lines cannot process nascent glycoprotein beyond a certain limit. In an earlier study, secreted recombinant human SC

was produced in mammalian cell lines using vaccinia virus recombinants (Cottet & Corthesy, 1997). However the yield of secreted recombinant human SC could not be improved by using a very potent T7/vaccinia virus hybrid system. It was suggested that saturation in the synthesis of ER chaperones such as calreticulin, calnexin, and PDI may limit the heterologous expression of SC in these cells.

In plants, it was already known that assembly of functional IgG molecules is very efficient (Hiatt *et al.*, 1989). Here we have extended the fidelity of plant assembly to include dimerisation of monomeric antibody by the J chain. We have also shown that plants engineered to simultaneously express an immunoglobulin light chain, hybrid IgG/A heavy chain, J chain and secretory component (SC) produce a chimaeric SIgA molecule. The transgenic plants are capable of expressing functional recombinant SIgA antibodies efficiently, at levels of up to 8 % total soluble protein. This level of expression is much higher than those previously reported for full-length IgG, which range from 0.35 % (Van Engelen *et al.*, 1994) to 1.3 % of total soluble protein in tobacco leaves (Ma *et al.*, 1994). We hypothesise that this increase in expression level may be due in part, to the increased resistance to proteolytic degradation *in planta*.

Using the transgenic plant material generated in this work, Hein and Wang have examined the efficiency of secretory component assembly with dimeric IgA by comparing the amounts of bound and unbound SC in plant extracts (unpublished, personal communication). Their estimates of approximately 50 % covalent assembly match those made in our laboratory and support the observation that multimeric protein assembly in plants is highly efficient.

The recombinant SIgA/G produced in transgenic tobacco plants is a hybrid molecule. It contains murine light, heavy and J chains and rabbit secretory component. Moreover, the recombinant plant SIgA/G molecule contained $\gamma 2/\alpha$ heavy chains, which contain an extra gamma C_H2 domain, compared with conventional antibodies. This did not appear to affect the assembly or antigen binding of the molecule. Retaining the γC_H2 domain might have a number of potential advantages in incorporating other functional regions; such as the complement-binding domain, in preserving a protein G binding site – which may simplify purification of the antibody – and in binding to macrophages. Some of these additional properties of SIgA/G may enhance the function of the complex in passive immunotherapy,

although under some circumstances these biological properties might be undesirable. In principle it should not be difficult to produce a SIgA/G antibody that lacks the γC_H2 domain in these cases.

Our findings confirm that in transgenic plants, as in mammals, the J chain is required for SC to assemble with antibody (Brandtzaeg & Prydz, 1984). A major proportion of the SC is associated with the dimeric form of IgA/G in plant extracts, as demonstrated by Western blotting. We confirmed that the rabbit secretory component (SC) was assembled with antibody in the SIgA/G complex but did not interfere with antigen recognition or binding. Despite the increased complexity of the plant SIgA/G molecule, the assembly of the antigen binding site was well preserved in plants, resulting in an affinity that is equivalent to that of the original IgG. This demonstrated that antigen recognition was unaffected by any differences in antibody structure, as a result of the complex nature of the SIgA/G molecule, or differences between plant and murine glycosylation.

A key difference between plants and mammals in the synthesis of SIgA lies in the site of assembly of the molecule. As described in the Literature review, in mammals dimeric IgA is synthesised and assembled in plasma cells, secreted extracellularly, whereupon it binds to the poly-immunoglobulin receptor (pIgR) produced by an epithelial cell. Following a process of transcytosis across the epithelial cell the pIgR is cleaved leaving SC attached to dimeric IgA. Thus two distinct cell types are involved. In plants, assembly of the secretory immunoglobulin takes place entirely within individual cells that co-express the four constituent chains (Ma *et al.*, 1995), and this probably occurs within the endoplasmic reticulum (Frigerio *et al.*, 2001). Thus in plants only single cells are required. This suggests that in mammals, the extracellular phase of dIgA assembly with secretory component is designed more to allow secretion of the antibody onto mucosal surfaces rather than to facilitate SIgA assembly itself.

In summary, there are many potential applications for secretory antibodies in mucosal immunotherapy and prevention. Although a number of strategies and expression systems have been used to produce secretory IgA antibodies, only plants appear to offer the potential for antibody production on the scale necessary to contemplate immunotherapeutic applications. An important breakthrough emerging from these studies is that the plant SIgA/G MAb prevented bacterial colonisation in a human clinical trial. This was the first

reported clinical trial of a secretory antibody in humans and a key step in realising the potential for pharmaceutical production using transgenic plants.

The levels of expression of SIgA/G already achieved represent one of the highest reported for a recombinant protein in plants. However it would clearly be advantageous to increase the level of antibody expression within the plant still further. The targeting of recombinant proteins to specific cellular locations (reviewed by Russell, 1999) is one such approach. With this in mind, more information on the precise site of antibody degradation may permit the most appropriate cellular location for maximum antibody accumulation to be selected. In addition, the assembly of secretory antibodies in plants has particular protein-folding and disulphide cross-linking requirements, and may involve plant homologues for molecular chaperones such as BiP and PDI, as discussed in Chapter 3. It is possible that in the future, strategies to optimise the level of antibody expression in plants may exploit the manipulation of chaperone levels. Once optimal expression strategies have been realised, then agricultural scale production of antibody-producing plants could realistically yield between thousands and hundreds of thousand kilograms per year.

DISCUSSION

DISCUSSION

Plant biotechnology has many applications that will be of benefit not only to the developed world, but particularly to developing countries. The first stage has been the introduction of genetically modified food crops, that will help to improve food productivity, reduce the use of agricultural chemicals and help to tackle the ever mounting problem of malnutrition. Other industrial crops, such as cotton, have followed and the next few years will see more and more conventional crops moving to genetically modified varieties around the world. Right from the start, the potential for using genetically modified plants for non-food uses was recognised, including the prospect of producing recombinant pharmaceuticals. The main attractions lay in the potential cost savings, the ability to produce on a large (agricultural) scale and the possibility of using edible transgenic plant material for the oral delivery of medicines.

From a commercial standpoint, monoclonal antibodies (MAbs) are generally regarded as the single most important class of protein in pharmaceutical development. They currently represent about 25 % of the clinical drug development pipeline and a recent Bank of America report has estimated that by 2010, there will be around 90 marketed monoclonal antibody products in the West. This will stretch production facilities to, and beyond, the limit. At present, 4 MAbs consume 75 % of the global capacity for antibody production. Furthermore, as indications for chronic use are developed, an order of magnitude increase in MAb requirements is expected. One of the major challenges that the industry faces, is how to meet this escalating demand for MAbs, that is projected to be up to 100 times current capacity by 2010. Currently, scalability is limited by the capacity of existing cell fermentation systems and the cost and 4-5 year lead time required for building and validating a new manufacturing facility.

Although heterologous expression systems exist for MAbs, none are regarded as ideal. In the most commonly used bacterial expression systems, the folding accuracy of recombinant proteins is usually low, and they are often stored in inclusion bodies. *E. coli* does not permit the formation of inter-domain disulphide bonds, and appears inefficient at producing full-length antibodies, moreover it does not carry out glycosylation. In bacterial expression systems as well as transgenic animals or mammalian cell culture, the co-purification of oncogenic and blood-borne pathogens is a significant concern in the

production and purification of antibodies, particularly when medium requirements incorporate derivations of animal products (Fischer *et al.*, 1999b).

For all these reasons, there was considerable interest when the expression of a monoclonal antibody in transgenic plants was first reported (Hiatt *et al.*, 1989). Nowadays, it is relatively straightforward to express a MAb in transgenic plants and many plant species have been utilised, including maize, rice, potato and alfalfa. Antibody expression in plants compares relatively favourably with more-established expression systems, however so far expression levels of proteins are still usually lower when compared with bacterial expression levels. Relatively little is known about the cellular mechanisms involved in plants, and a better understanding of these processes is likely to lead to new strategies to optimise antibody expression, as well as the identification of new multimeric proteins that can be expressed in plants.

Our study began with an investigation into how the plant ER responds to the expression of immunoglobulin chains as heterologous secretory proteins. We studied the involvement of BiP and calreticulin in the folding and assembly of recombinant immunoglobulin heavy and light chains in transgenic plants. Our finding that BiP is actively involved in antibody processing, might explain the efficiency and relatively high yield of functional antibody production that can be achieved in plants. It will be of great interest to identify other chaperones within the plant endomembrane system that take part in MAb folding and assembly, and to try to discover bottlenecks in the pathway that could be alleviated by chaperone engineering. The feasibility of this approach has already been adopted for BiP and calreticulin in tobacco (Denecke *et al.*, 1993; 1995).

The next step was to determine the extent of glycosylation of plant derived MAbs. We showed that N-linked glycans were added to the same sites that were used by the parent murine hybridoma. A subsequent detailed analysis using the antibody purified in this work showed that whereas the mouse MAb contained only complex-type glycans, the plant version was more heterogenous in glycosylation, containing five high-mannose structures comprising 40 % of the glycans, and three complex structures, comprising 60 %. These differences in the glycosylation pattern of the plant version of Guy's 13 MAb had no effect on antigen binding or specificity, but it has been suggested that they might lead to immunogenic or even allergenic reactions, upon clinical administration (Bardor *et al.*, 2003). Plant derived monoclonal antibodies from this study were utilised to study the

potential immunogenicity of the plant glycans following both mucosal administration in human subjects and parenteral immunisation in mice (Ma *et al.*, 1998; Chargelegue *et al.*, 2000). No evidence for immune recognition was detected in either case. Nevertheless, although it may seem that plant glycans do not present an immunological challenge when presented on a MAb, this will remain a potential area for concern. For this reason, research into engineering of the glycosylation pathway in plants may be a pragmatic approach to avoiding the issue altogether.

Having passed through the Golgi apparatus, the default pathway for IgG in plants is secretion (Frigerio *et al.*, 2000). This may not be the optimal end-result for recombinant MAbs. Other groups have studied the retention of immunoglobulins in the ER (Conrad & Fiedler, 1998), and here we investigated the possibility of targeting and retention of IgG in the plasma membrane. Many eukaryotic membrane proteins have proved difficult to express in recombinant systems. But in plants, we have demonstrated that the native membrane association sequence from B cells (Tyler *et al.*, 1982), can act as a membrane targeting sequence in plants. Unlike retention in the ER, an increase in accumulation levels was not observed above that achieved in plants that secreted IgG. As an extension to these findings, we also demonstrated that a second type of mammalian membrane protein - the seven transmembrane chemokine receptor CCR-5 - could be expressed in the plant plasma membrane. This demonstrated that a more generalised approach can be used for expressing membrane proteins in plants.

Utilising membrane retention sequences may have several applications. Firstly, controlling secretion of the recombinant protein is likely to have a beneficial environmental impact, by preventing leakage of the protein into the rhizosphere or other external plant fluids. Secondly, it might lead to easier antibody purification strategies, as the antibody can be initially purified in a non-soluble fraction. Finally, there may be advantages for antibodies intended for *in planta* purposes. These applications might comprise the immunomodulation of plant antigens or proteins of non-plant origin, in order to manipulate the plant's metabolism, and intra- or extracellular immunisation (Tavladoraki *et al.*, 1993), to achieve resistance against plant pathogens.

In the final part of the thesis, we have described a further development of our studies, from expressing the simplest immunoglobulin tetrameric complex to the more complex decameric structure that comprises a secretory antibody. We demonstrated that plants

engineered to simultaneously co-express an immunoglobulin light chain, a hybrid IgG-A heavy chain, a J chain and a secretory component (SC) could produce a chimeric SIgA molecule. The recombinant plant SIgA-G was targeted to the endoplasmic reticulum, where it accumulated at levels of up to 8 % of soluble plant protein. These studies ultimately led to the use of this secretory antibody in a clinical trial, in which it was demonstrated for the first time, that a plant-derived recombinant pharmaceutical was efficacious in humans (Ma *et al.*, 1998).

This work has investigated not only some of the cellular processes involved in antibody production in plants, but it has also explored some novel applications for which this knowledge can be applied. An understanding of how multimeric protein complexes assemble and the capacity of plants to achieve this, would open the way for the design and engineering of new complex proteins, built around a basic immunoglobulin structure. It may be possible to envisage many other therapeutic molecules which are amenable to expression in plants, for which, like secretory antibody, plants represent the only practical system for production.

REFERENCES

Ahnen, D.J., J.R. Singleton, T.C. Hoops and T.M. Kloppel. 1986. Posttranslational processing of secretory component in the rat jejumum by a brush border metalloprotease. J. Clin. Invest. **77**: 1841-1848.

Ailor, E. and M.J. Betenbaugh. 1998. Overexpression of a cytosolic chaperone to improve solubility and secretion of a recombinant IgG protein in insect cells. Biotechnol. Bioeng. **58**: 196-203.

Ailor, E. and M.J. Betenbaugh. 1999. Modifying secretion and post-translational processing in insect cells. Curr. Opin. Biotechnol. **10**: 142-151.

Alkhatib, G., C. Combadiere, C.C. Broder, Y. Feng, P.E. Kennedy, P.M. Murphy and E.A. Berger. 1996. CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. Science **272**: 1955-1958.

Anderson, J.V., Q-B. Li, D.W. Haskell and C.L. Guy. 1994. Structural organisation of the spinach endoplasmic reticulum-luminal 70-Kilodalton heat-shock cognate gene and expression of 70-Kilodalton heat-shock genes during cold acclimation. Plant Physiol. **104**: 1359-1370.

Atkin, J.D., R.J. Pleass, R.J. Owens and J.M. Woof. 1996. Mutagenesis of the human IgA1 heavy chain tailpiece that prevents dimer assembly. J. Immunol. **157**: 156-159.

Bakker, H., M. Bardor, J.W. Molthoff, V. Gomord, I. Elbers, L.H. Stevens, W. Jordi, A. Lommen, L. Faye, P. Lerouge and D. Bosch. 2001. Galactose-extended glycans of antibodies produced by transgenic plants. Proc. Natl. Acad. Sci. USA **98**: 2899-2904.

Bakos, M.A., A. Kurosky and R.M. Goldblum. 1991. Characterisation of a critical binding site for human polymeric Ig on secretory component. J. Immunol **147**: 3419-3426.

Bardor, M., C. Faveeuw, A.C. Fitchette, D. Gilbert, L. Galas, F. Trottein, L. Faye and P. Lerouge. 2003. Immunoreactivity in mammals of two typical plant glyco-epitopes, core alpha (1,3)-fucose and core xylose. Glycobiology **13**: 427-434.

Barnes, W.M. 1990. Variable patterns of expression of luciferase in transgenic tobacco leaves. Proc. Natl. Acad. Sci. USA **87**: 9183-9187.

Baselga, J., D. Tripathy, J. Mendelsohn, S. Baughman, C.C. Benz, L. Dantis, N.T. Sklarin, A.D. Seidman, C.A. Hudis, J. Moore, P.P. Rosen, T. Twaddell, I.C. Henderson, L. Norton. 1996. Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. J. Clin Oncol. **14**:737-44.

Baumal, R., B.K. Birshtein, P. Coffino and M.D. Scharff. 1973. Mutations in immunoglobulin-producing mouse myeloma cells. Science **182**: 164-166.

Beighton, D., R.R. Russell and R.A. Whiley. 1991. A simple biochemical scheme for the differentiation of *Streptococcus mutans* and *Streptococcus sobrinus*. Caries Res. **25**: 174-178.

Bendelac, A. and D.T. Fearon. 1997. Innate immunity – innate pathways that control acquired immunity. Curr. Opin. Immunol. 9: 1-3.

Benfey, P.N. and N.-H. Chua. 1989. The cauliflower mosaic virus 35S promoter: combinatorial regulation of transcription in plants. Science 250: 959-966.

Benvenuto, E., R.J. Ordas, R. Tavazza, G. Ancora, S. Biocca, A. Cattaneo and P. Galeffi. 1991. 'Phytoantibodies' a general vector for the expression of immunoglobulin domains in transgenic plants. Plant Mol. Biol. 17: 865-874.

Berdoz, J., C.T. Blanc, M. Reinhardt, J-P. Kraehenbuhl and B. Corthesy. 1999. *In vitro* comparison of the antigen-binding and stability properties of the various molecular forms of IgA antibodies assembled and produced in CHO cells. Proc. Natl. Acad. Sci. USA 96: 3029-3034.

Bergeron, J.M., M.B. Brenner, D.Y. Thomas and D.B. Williams. 1994. Calnexin: a membrane-bound chaperone of the endoplasmic reticulum. Trends Biochem. Sci. 19: 124-128.

Bergman, L.W. and W.M. Kuehl. 1979. Co-translational modification of nascent immunoglobulin heavy and light chains. J. Supramol. Struct. 11: 9-24.

Berridge, M.J. 1993. Inositol trisphosphate and calcium signalling. Nature 361: 315-325.

Bevan, M., W.M. Barnes and M.D. Chilton. 1983. Structure and transcription of the nopaline synthase gene region from T-DNA. Nucleic Acid Res. 11: 369-385.

Birch, R.G. 1997. Plant transformation: problems and strategies for practical application. Annu. Rev. Plant Physiol. & Plant Mol. Biol. 48: 297-326.

Blond-Elguindi, S.B., S.E. Cwirla, W.J. Dower, R.J. Lipshutz, S.R. Sprang, J.F. Sambrook and M-J.H. Gething. 1993a. Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of BiP. Cell 75: 717-728.

Blond-Elguindi, S.B., A.M. Fourie, J.F. Sambrook and M-J.H. Gething. 1993b. Peptide-dependent stimulation of the ATPase activity of the molecular chaperone BiP is the result of conversion of oligomers to active monomers. J. Biol. Chem. 268: 12730-12735.

Bogers, W.M.J.M., L.A. Bergmeier, J. Ma, H. Oostermeijer, Y. Wang, C.G. Kelly, P. ten Haaft, M. Singh, J.L. Heeney and T. Lehner. 2003. AIDS (In press).

Bole, D.G., L.M. Hendershot and J.F. Kearney. 1986. Post-translational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. J. Cell Biol. 102: 1558-1566.

Bookman, M.A. 1998. Biological therapy of ovarian cancer: current directions. Semin. Oncol. 25: 381-396.

Boss, M.A., J.H. Kenten, C.R. Wood and J.S. Emtage. 1984. Assembly of functional antibodies from immunoglobulin heavy and light chains synthesised in *E. coli*. Nucleic Acids Res. 12 : 3791-3806.

Boston, R.S., P.V. Viitanen and E. Vierling. 1996. Molecular chaperones and protein folding in plants. Plant Mol. Biol. **32**: 191-222.

Braakman, I., H. Hoover-Liny, K.R. Wagner and A. Helenius. 1991. Folding of influenza hemagglutinin in the endoplasmic reticulum. J. Cell Biol. **114**: 401-411.

Braakman, I., J. Helenius and A. Helenius. 1992. Manipulating disulphide bond formation and protein folding in the endoplasmic reticulum. EMBO J. **114**: 1717-1722.

Brandtzaeg, P. 1970. Unfolding of human secretory immunoglobulin A. Immunochemistry **7**: 127-130.

Brandtzaeg, P., I. Fjellanger and I. Gjeruldsen. 1970. Salivary secretions from individuals with normal or low levels of serum immunoglobulins. Scand. J. Haematol. **12**: 3-83.

Brandtzaeg, P. 1994. Distribution and characterisation of mucosal immunoglobulin-producing cells. In: P.L. Ogra, J. Mestecky, M.E. Lamm, W. Strober, J.R. McGhee, J. Bienenstock (eds.). Handbook of mucosal immunity. Academic Press, New York, pp. 251-259.

Brandtzaeg P. and H. Prydz. 1984. Direct evidence for an integrated function of J chain and secretory component in epithelial transport of immunoglobulins. Nature **311**: 71-73.

Breedveld, F.C. 2000. Therapeutic monoclonal antibodies. Lancet 355: 735-740.

Brown, W.R., R.W. Newcomb and K. Ishizaka. 1970. Proteolytic degradation of exocrine and serum immunoglobulins. J. Clin. Invest. **49**: 1374-1380.

Bulleid, N.J. and R.B. Freedman. 1988. Defective co-translational formation of disulphide bonds in protein disulphide isomerase-deficient microsomes. Nature **335**: 649-651.

Cabanes-Macheteau, M., A-C. Fitchette-Laine, C. Loutelier-Bourhis, C. Lange, N.D. Vine, J.K-C. Ma, P. Lerouge and L. Faye. 1999. N-Glycosylation of a mouse IgG expressed in transgenic tobacco plants. Glycobiology **9**: 365-372.

Carayannopoulos, L., E.E. Max and J.D. Capra. 1994. Recombinant human IgA expressed in insect cells. Proc. Natl. Acad. Sci. USA **91**: 8348-8352.

Carlsson, L. and E. Lazarides. 1983. ADP-ribosylation of the M_r 83,000 stress-inducible and glucose-related protein in avian and mammalian cells: modulation by heat shock and glucose starvation. Proc. Natl. Acad. Sci. USA **80**: 4664-4668.

Casanova, J.E., P.P. Breitfield, S.A. Ross and K.E. Mostov. 1990. Phosphorylation of the polymeric immunoglobulin receptor is required for its efficient transcytosis. Science **248**: 742-745.

Casanova, J.E., G. Apodaca and K.E. Mostov. 1991. AN autonomous signal for basolateral orting in the cytoplasmic domain of the polymeric immunoglobulin receptor. Cell **66**: 65-75.

Challacombe, S.J. and T. Lehner. 1976. Serum and salivary antibodies to cariogenic bacteria in man. J. Dent. Res. **55**: Spec No: C139-148.

Chan, M.T., H.H. Chang, S.L. Ho, W.F. Tong and S.M. Yu. 1993. Agrobacterium-mediated production of transgenic rice plants expressing a chimeric alpha-amylase promoter/beta-glucoronidase gene. Plant Mol. Biol. **22**: 491-506.

Chargelegue, D., N.D. Vine, C.J. van Dolleweerd, P.M.W. Drake and J.K-C. Ma. 2000. A murine monoclonal antibody produced in transgenic plants with plant-specific glycans is not immunogenic in mice. Transgen. Res. **9**: 187-194.

Chen, F. and P.M. Hayes. 1994. Nucleotide sequence and developmental expression of duplicated genes encoding protein disulphide isomerase in barley. Plant Physiol. **106**: 1705-1706.

Chen, F., P.M. Hayes, D.M. Mulrooney and A. Pan. 1994. Identification and characterisation of cDNA clones encoding plant calreticulin in barley. Plant Cell **6**: 835-843.

Cheng, M.Y., F.-U.Hartl, J. Martin, R.A. Pollock, F. Kalousek, W. Neupert, E.M. Hallberg, R.L. Hallberg and A.L. Horwich. 1989. Mitochondrial heat-shock protein hsp60 is essential for assembly of proteins imported into yeast mitochondria. Nature **337**: 620-625.

Chintalacharuvu, K.R., A.S. Tavill, L.N. Louis, J.P. Vaerman, M.E. Lamm and C. Kaetzel. 1994. Disulphide bond formation between dimeric immunoglobulin A and the polymeric immunoglobulin receptor during hepatic transcytosis. Hepatol. **19**: 162-173.

Chintalacharuvu, K.R. and S.L. Morrison. 1997. Production of secretory immunoglobulin A by a single mammalian cell. Proc. Natl. Acad. Sci. USA **94**: 6364-6368.

Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanante-phenol-chloroform extraction. Anal. Biochem. **162**: 156-159.

Christou, P. 1995. Particle bombardment. Meth. Cell Biol. **50**: 375-382.

Chuang, P.D. and S.L. Morrison. 1997. Elimination of N-linked glycosylation sites from the human IgA1 constant region. J. Immunol. **158**: 724-732.

Co, M.S., M. Deschamps, R.J. Whitley and C. Queen. 1991. Humanised antibodies for antiviral therapy. Proc. Natl. Acad. Sci. USA **88**: 2869-2873.

Coleman, C.E., M.A. Lopes, J.W. Gillikin, R.S. Boston and B.A. Larkins. 1995. A defective signal peptide in the maize high-lysine mutant *floury 2*. Proc. Natl. Acad. Sci. USA **92**: 6828-6831.

Collins, R.A., T.B. Ng, W.P. Fong, C.C. Wan and H.W. Yeung. 1998. Removal of polyphenolic compounds from aqueous plant extracts using polyamide minicolumns. Biochem. Mol. Biol. Int. **45**: 791-796.

Conrad, U. and U. Fiedler. 1998. Compartment-specific accumulation of recombinant immunoglobulins in plant cells: an essential tool for antibody production and immunomodulation of physiological functions and pathogen activity. Plant Mol. Biol. **38**: 101-109.

Corthesy, B. 1997. Recombinant secretory IgA for immune intervention against mucosal pathogens. Biochem. Soc. Trans. **25**: 471-475.

Corthesy, B. and J-P. Kraehenbuhl. 1999. Antibody-mediated protection of mucosal surfaces. Curr. Top. Microbiol. Immunol. **236**: 93-111.

Cosimi, A.B. 1983. Diagnostic and therapeutic applications of monoclonal antibodies to human T-cell subsets in renal transplant recipients. Urol. Clin. North Am. **10**: 289-299.

Cottet, S. and B. Corthesy. 1997. Cellular processing limits the heterologous expression of secretory component in mammalian cells. Eur. J. Biochem. **246**: 23-31.

Coughlan, S.J., C. Hastings, J.R. Winfrey. 1996. Molecular characterisation of plant endomembrane reticulum: identification of protein disulphide-isomerase as the major reticuloplasmin. Eur. J. Biochem. **235**: 215-244.

Coughlan, S.J., C. Hastings and R. Winfrey. 1997. Cloning and characterisation of the calreticulin gene from *Ricinus communis* L. Plant Mol. Biol. **34**: 897-911.

Crofts, A.J., N. Leborgne-Castel, M. Pesca, A. Vitale and J. Denecke. 1998. BiP and calreticulin form an abundant complex that is independent of endoplasmic reticulum stress. Plant Cell **10**: 813-823.

Crofts, A. and J. Denecke. 1998. Calreticulin and calnexin in plants. Trends Plant Sci. **3**: 396-399.

Crottet, P. and B. Corthesy. 1998. Secretory component delays the conversion of secretory IgA into antigen-binding competent F(ab')2: a possible implication for mucosal defence. J. Immunol. **161**: 5445-5453.

Dai, Y. and C. Wang. 1997. A mutant truncated protein disulphide isomerase with no chaperone activity. J. Biol. Chem. **272**: 27572-27576.

D'Amico, L., B. Valsasina, M.G. Dauninati, M.S. Fabbrini, G. Nitti, R. Bollini, A. Ceriotti and A. Vitale. 1992. Bean homologs of the mammalian glucose-regulated proteins: induction by tunicamycin and interaction with newly synthesised seed storage proteins in the endoplasmic reticulum. Plant J. **2**: 443-455.

Danilczyk, U.G., M.F. Cohen-Doyle and D.B. Williams. 2000. Functional relationship between calreticulin, calnexin, and the endoplasmic reticulum luminal domain of calnexin. J. Biol. Chem. **275**: 13089-13097.

Davidson, D.J., M.J. Fraser and F.J. Castellino. 1990. Oligosaccharide processing in the expression of human plasminogen cDNA by lepidopteran insect (*Spodoptera frugiperda*) cells. Biochem. **29**: 5584-5590.

De Greve, H., P. Dhaese, J. Seurinck, M. Lemmers, M. Van Montagu and J. Schell. 1983. Nucleotide sequence and transcript map of the *Agrobacterium tumefaciens* Ti plasmid-encoded octopine synthase gene. J. Mol. Appl. Genet. **1**:499- 511.

Deisenhofer, J. 1981. Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from Staphylococcus aureus at 2.9- and 2.8-A resolution. Biochem. **20**: 2361-2370.

Deitcher, D.L. and K.E. Mostov. 1986. Alternate splicing of rabbit polymeric immunoglobulin receptor. Mol. Cell Biol. **6**: 2712-2715.

Delacroix, D.L., C. Dive, J.C. Rambaud and J.P. Vaerman. 1982. IgA subclasses in various secretions and in serum. Immunol. 47: 383-385.

Denecke, J., M.H.S. Goldman, J. Demolder, J. Seurinck and J. Botterman. 1991. The tobacco luminal binding protein is encoded by a multigene family. Plant Cell 3: 1025-1035.

Denecke, J. L.E. Carlsson, S. Vidal, A-S. Hoglund, B. Ek, M.J. van Zeijl, K.M.C. Sinjorgo and E.T. Palva. 1995. The tobacco homolog of mammalian calreticulin is present in protein complexes *in vivo*. Plant Cell 7: 391-406.

De Witt, N.D., B. Hong, M.R. Sussman and J.F. Harper. 1996. Targeting of two Arabidopsis (H+) ATPase isoforms to the plasma membrane. Plant Physiol. **112**: 833-844.

Di Rita, V.J. and S.B. Gelvin. 1987. Deletion analysis of the mannopine synthase gene promoter in sunflower crown gall tumors and *Agrobacterium tumefaciens* Mol. Gen Genet. **207**: 233-241.

Donadel, G., A. Calabro, G. Sigounas, V.C. Hascall, A.L. Notkins and N. Harindranath. 1994. Human polyreactive and monoreactive antibodies: effect of glycosylation on antigen binding. Glycobiol. **4**: 491-496.

Donson, J., C.M. Kearney, M.E. Hilf, W.O. Dawson. 1991. Systemic expression of a bacterial gene by a tobacco mosaic virus-based vector. Proc. Natl. Acad. Sci. USA **88**: 7204-7208.

Dorner, A.J., L.C. Wasley and R.J. Kaufman. 1990. Protein dissociation from GRP78 and secretion are blocked by depletion of cellular ATP levels. Proc. Natl. Acad. Sci. USA **87**: 429-432.

Dorner, A.J., L.C. Wasley and R.J. Kaufman. 1992. Overexpression of GRP78 mitigates stress induction of glucose regulated proteins and blocks secretion of selective proteins in Chinese hamster ovary cells. EMBO J. **11**: 1563-1571.

Drakakaki, G., P. Christou and E. Stoger. 2000. Constitutive expression of soybean ferritin cDNA in transgenic wheat and rice results in increased iron levels in vegetative tissues but not in seeds. Transgenic Res. **9**: 445-452.

Dresselhaus, T., C. Hagel, H. Lorz and E. Kranz. 1996. Isolation of a full-length cDNA encoding calreticulin from a PCR library of *in vitro* zygotes from maize. Plant Mol. Biol. **31**: 23-34.

Driouich, A., L. Faye and L.A. Staehelin. 1993. The plant Golgi apparatus: a factory for complex polysaccharides and glycoproteins. Trends Biochem. Sci. **18**: 210-214.

Dul, J.L. and Y. Argon. 1990. A single amino acid substitution in the variable region of the light chain specifically blocks immunoglobulin secretion. Proc. Natl. Acad. Sci. USA **87**: 8135-8139.

Endo, T., J. Mestecky, R. Kulhavy and A. Kobata. 1994. Carbohydrate heterogeneity of human myeloma proteins of the IgA1 and IgA2 subclasses. Mol. Immunol. **31**: 1415- 1422.

Eng, T. and W. Butler (eds.). 1997. The Hidden Epidemic: Confronting Sexually Transmitted Diseases. Washington, D.C.. National Academy Press.

Faye, L., V. Gomord, A. Fitchette-Laine and M.J. Chrispeels. 1993. Affinity purification of antibodies specific for Asn-linked glycans containing alpha (1,3)-fucose or beta (1,2)-xylose. Anal. Biochem. **209**: 104-108.

Fiedler, K. and K. Simmons. 1995. The role of N-glycans in the secretory pathway. Cell **81**: 309-312.

Filler, S.J., R.L. Gregory, S.M. Michalek, J. Katz and J.R. McGhee. 1991. Effect of immune bovine milk on *Streptococcus mutans* in human dental plaque. Archs. Oral Biol. **36**: 41-47.

Fischer, R., Y.C. Liao and J. Drossard. 1999a. Affinity-purification of a TMV-specific recombinant full-size antibody from a transgenic tobacco suspension culture. J. Immunol. Meth. **226**: 1-10.

Fischer, R., Y-C. Liao, K. Hoffmann, S. Schillberg and N. Emans. 1999b. Molecular farming of recombinant antibodies in plants. Biol. Chem. **380**: 825-839.

Fleming, D.T., G.M. McQuillian, R.E. Johnson, A.J. Nahmias, S.O. Aral and F.K. Lee and M.E. St Louis. 1997. Herpes simplex virus type 2 in the United States, 1976 to 1994. N. Eng. J. Med. **337**: 1105-1111.

Flynn, G.C., T.G. Chappell and J.E. Rothman. 1989. Peptide binding and release by proteins implicate as catalysts of protein assembly. Science **245**: 385-390.

Flynn, G.C., J. Pohl, M.T. Flocco and J.E. Rothman. 1991. Peptide-binding specificity of the molecular chaperone BiP. Nature **353**: 726-730.

Fontes, E.B.P., B.B. Shank, R.L. Wrobel, S.P. Moose, G.R. O'Brian, E.T. Wurtzel and R.S. Boston. 1991. Characterisation of an immunoglobulin binding protein homolog in the maize *floury-2* endosperm mutant. Plant Cell **3**: 483-496.

Fraley, R.T., S.G. Rogers, R.B. Horsch, P.R. Sanders, J.S. Flick, S.P. Adams, M.L. Bittner, L.A. Brand, C.L. Fink, J.S. Fry, G.R. Galluppi, S.B. Goldberg, N.L. Hoffmann and S.C. Woo. 1983. Expression of bacterial genes in plant cells. Proc. Natl. Acad. Sci. USA. **80**: 4803-4807.

Freedman, R.B. 1989. Protein disulphide isomerase: multiple roles in the modification of nascent secretory proteins. Cell **57**: 1069-1072.

Freedman, R.B., T.R. Hirst and M.F. Tuite. 1994. Protein disulphide isomerase: building bridges in protein folding. Trends Biochem. Sci. **19**: 331-336.

Freiden, P.J., J.R. Gaut and L.M. Hendershot. 1992. Interconversion of three differentially modified and assembled forms of BiP. EMBO J. **11**: 63-70.

Frigerio, L., N.D. Vine, E. Pedrazzini, M.B. Hein, F. Wang, J.K-C. Ma and A. Vitale. 2000. Assembly, secretion and vacuolar delivery of a hybrid immunoglobulin in plants. Plant Physiol. **123**: 1483-1493.

Frigerio, L., A. Pastres, A. Prada and A. Vitale. 2001. Influence of KDEL on the fate of trimeric or assembly-defective phaseolin: selective use of an alternative route to vacuoles. Plant Cell **13**: 1109-1126.

Frutiger, S., G.J. Hughes, C. Fonck and J.C. Jaton. 1987. High and low molecular weight rabbit secretory components. Evidence for the deletion of the second and third domains in the smaller polypeptide. J. Biol. Chem. **262**: 1712-1715.

Galbraith, D.W. 1989. Analysis of higher plants by flow cytometry and cell sorting. Int. Rev. Cytol. **116**: 165-228.

Garcia-Pardo, A., M.E. Lamm, A.G. Plaut and B. Frangione. 1981. J chain is covalently bound to both monomer subunits in human secretory IgA. J. Biol. Chem. **256**: 11734-11738.

Gardner, A.M., S. Aviel and Y. Argon. 1993. Rapid degradation of an unassembled immunoglobulin light chain is mediatedby a serine protease and occurs in a pre-Golgi compartment. J. Biol. Chem. **268**: 25940-25947.

Gething, M-J. and J. Sambrook. 1992. Protein folding in the cell. Nature **355**: 33-45.

Gething, M-J. . 1999. Role and regulation of the ER chaperone BiP. Seminars Cell Dev. Biol. **10**: 465-472.

Gilbert, H.F. 1994. Protein chaperones and protein folding. Curr. Opin. Biotechnol. **5**: 534-539.

Gilbert, J.V., A.G. Plaut, B. Longmaid and M.E. Lamm. 1983. Inhibition of microbial IgA proteases by human secretory IgA and serum. Mol. Immunol. **20**: 1039-1049.

Goldberger, R.F., C.J. Epstein and C.B. Anfinsen. 1963. Acceleration of the reactivation of reduced bovine pancreatic ribonuclease by a microsomal system from rat liver. J. Biol. Chem. **238**: 628-635.

Goloubinoff, P., J.T. Christeller, A.A. Gatenby and G.H. Lorimer. 1989. Reconstitution of active dimeric ribulose bisphosphate carboxylase from an unfolded state depends on two chaperonin proteins and Mg-ATP. Nature **342**: 884-889.

Gomord, V., A.C. Fitchette-Laine, L.A. Denmat, D. Michaud and L. Faye. 1998. Production of foreign proteins in tobacco cells. In: C. Cunningham and A.J. Porter (eds.). Methods in Molecular Biotechnology. Humana Press Inc., New Jersey, pp. 155-164.

Goode, J.H., S.B. Settlage, R.F. Wilson and R.E. Dewey. 1995. Isolation of a calnexin homolog from developing soybean seeds. Plant Physiol. **108**: 1341.

Goto, F., T. Yoshihara, N. Shigemoto, S. Toki and F. Takaiwa. 1999. Iron fortification of rice seed by the soybean ferritin gene. Nature Biotechnol. **17**: 282-286.

Haas, I.G. 1991. BiP – a heat shock protein involved in immunoglobulin chain assembly. Curr. Top. Microbiol. Immunol. **167**: 71-82.

Haas, I.G. and M. Wabl. 1983. Immunoglobulin heavy chain binding protein. Nature **306**: 387-389.

Haas, I.G. and T. Meo. 1988. cDNA cloning of the immunoglobulin heavy chain binding protein. Proc. Natl. Acad. Sci. USA 85: 2250-2254.

Hadlington, J.L., A. Santoro, J. Nutall, J. Denecke, J. K-C. Ma, A. Vitale and L. Frigerio. 2003. The C-terminal extension of a hybrid immunoglobulin A/G heavy chain is responsible for its' Golgi-mediated sorting to the vacuole. Mol. Biol. Cell 14: 2592-2602.

Hamm, C.W., C. Heeschen, B. Goldmann, A. Vahanian, J. Adgey, C.M. Miguel, W. Rutsch, J. Berger, J. Kootstra and M.L. Simoons. 1999. Benefit of abciximab in patients with refractory unstable angina in relation to serum troponin T levels. N. Eng. J. Med. 340: 1623-1629.

Hamman, B.D., L.M. Hendershot and A.E. Johnson. 1998. BiP maintains the permeability barrier of the ER membrane by sealing the lumenal end of the translocon pore before and early in translocation. Cell 92: 747-758.

Hammond, C., I. Braakman and A. Helenius. 1994. Role of N-linked oligosaccharide recognition, glucose trimming and calnexin in glycoprotein folding and quality control. Proc. Natl. Acad. Sci. USA 91: 913-917.

Hammond, C. and A. Helenius. 1994. Folding of VSV G protein: sequential interaction with BiP and calnexin. Science 266: 456-458.

Hammond, C. and A. Helenius. 1995. Quality control in the secretory pathway. Curr. Opin. Cell Biol. 7: 523-529.

Hartl, F.U. 1996. Molecular chaperones in cellular protein folding. Nature 381: 571-580.

Haseman, C.A. and J.D. Capra. 1990. High-level production of a functional immunoglobulin heterodimer in a baculovirus system. Proc. Natl. Acad. Sci. USA 87: 3942-3946.

Hassan, A.M., C. Wesson and W.R. Trumble. 1995. Calreticulin is the major Ca^{2+}-storage protein in the endoplasmic reticulum of the pea plant (*Pisum sativum*). Biochem. Biophys. Res. Commun. 211: 54-59.

Hebert, D.N., B. Foellmer and A. Helenius. 1995. Glucose trimming and reglucosylation determine glycoprotein association with calnexin in the endoplasmic reticulum. Cell 81: 425-433.

Hebert, D.N., B. Foellmer and A. Helenius. 1996. Calnexin and calreticulin promote folding, delay oligomerisation and suppress degradation of influenza hemagglutinin in microsomes. EMBO J. 15: 2961-2968.

Hebert, D.N., J.X. Zhang, W. Chen, B. Foellmer and A. Helenius. 1997. The number and location of glycans on influenza hemagglutinin determines folding and association with calnexin and calreticulin. J. Cell Biol. 139: 613-623.

Hein, M.B., Y. Tang, D.A. McLeod, K.D. Janda and A.C. Hiatt. 1991. Evaluation of immunoglobulins from plant cells. Biotechnol. Prog. 7: 455-461.

Helenius, A., E.S. Trombetta, D.N. Herbert and J.F. Simons. 1997. Calnexin, calreticulin and the folding of glycoproteins. Trends Cell Biol. 7: 193-200.

Helenius, A. and M. Aebi. 2001. Intracellular functions of N-linked glycans. Science 291 (5512): 2364-2369.

Hellman, R., M. Vanhove, A. Lejeune, F.J. Stevens and L.M. Hendershot. 1999. The *in vivo* association of BiP with newly synthesised proteins is dependent on the rate and stability of folding and not simply on the presence of sequences that can bind to BiP. J. Cell Biol. **144**: 21-30.

Hendershot, L., D. Bole, G. Kohler and J.F. Kearney. 1987. Assembly and secretion of heavy chains that do not associate posttranslationally with immunoglobulin heavy chain-binding protein. J Cell Biol. **104**: 761-767.

Hendershot, L., J. Wei, J. Gaut, J. Melnick, S. Aviel and Y. Argon. 1996. Inhibition of immunoglobulin folding and secretion by dominant negative BiP ATPase mutants. Proc. Natl. Acad. Sci. USA **93**: 5269-5274.

Hendrick, J.P. and F.U. Hartl. 1993. Molecular chaperone functions of heat-shock proteins. Annu. Rev. Biochem. **62**: 349-384.

Herscovics, A.O. and P. Orlean. 1993. Glycoprotein biosynthesis in yeast. FASEB J. **7**: 540-550.

Hiatt, A.C., R. Cafferkey and K. Bowdish. 1989. Production of antibodies in transgenic plants. Nature **342**: 76-78.

Hitzeman, R.A., D.W. Leung, L.J. Perry, W.J. Kohr, H.L. Levine and D.V. Goeddel. 1983. Secretion of human interferons by yeast. Science **219**: 620-625.

Holmgren, J., A.M. Svennerholm and O. Ouchterlony. 1972. Antibodies to endotoxin from *Vibrio cholerae* measured by bactericidal and indirect haemagglutination tests. Differences in immunoglobulin classes and specificity. J. Immunol. Meth. **4**: 329-340.

Horsch, R.B., J.E. Fry, N.C. Hoffman, D. Eichholtz, S.G. Rogers and R.T. Fraley. 1985. Science **227**:1229-1231.

Horwitz, A.H., C.P. Chang, M. Better, K.E. Hellstrom and R.R. Robinson. 1988. Secretion of functional antibody and Fab' fragment from yeast cells. Proc. Natl. Acad. Sci. USA **85**: 8678-8682.

Hsu, T-A, J.J. Eiden, P. Bourgarel, T. Meo and M.J. Betenbaugh. 1994. Effects of co-expressing chaperone BiP on functional antibody production in the baculovirus system. Protein Expr. Pur. **5**: 595-603.

Hu, W-S. and J.G. Aunins. 1997. Large-scale mammalian cell culture. Curr. Opin. Biotechnol. **8**: 148-153.

Huang, L., A.E. Franklin and N.E. Hoffman. 1993. Primary structure and characterisation of an Arabidopsis thaliana calnexin-like protein. J. Biol. Chem. **268**: 6560-6566.

Humphreys, D.P., N. Weir, A. Lawson, A. Mountain and P.A. Lund. 1996. Co-expression of human protein disulphide isomerase (PDI) can increase the yield of an antibody Fab' fragment expressed in *Escherichia coli*. FEBS Lett. **380**: 194-197.

Hunkapiller T. and L. Hood. 1989. Diversity of the immunoglobulin gene superfamily. Adv. Immunol. **44**: 1-63.

Huston, J.S., D. Levinson, M. Mudgett-Hunter, M-S. Tai, J. Novotny, M.N. Margolies, R.J. Ridge, R.E. Bruccoleri, E. Haber, R. Crea and H. Oppermann. 1988. Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **85**: 5879-5883.

Hwang, C., A.J. Sinskey, and H.F. Lodish. 1992. Oxidised redox state of glutathione in the endoplasmic reticulum. Science **257**: 1496-1502.

Ishida, Y., H. Saito, S. Ohta, Y. Hiei, T. Komari and T. Kumashiro. 1996. High efficiency transformation of maize (*Zea mays L.*) mediated by *Agrobacterium tumefaciens*. Nature Biotechnol. **14**: 745-750.

James, D.C., R.B. Freedman, M. Hoare, O.W. Ogonah, B.C. Roonez, O.A. Larionov, V. Dobrovolsky, O.V. Lagutin and N. Jenkins. 1995. N-glycosylation of recombinant human interferon γ produced in different animal expression systems. Bio-Technology **13**: 592-596.

Jarvis, D.L., C. Oker-Blom and M.D. Summers. 1990. Role of glycosylation in the transport of recombinant glycoproteins through the secretory pathway of lepidopteran insect cells. J. Cell Biochem. **42**: 181-191.

Jeenes, D.J., D.A. McKenzie, I.N. Roberts and D.B. Archer. 1991. Heterologous protein expression by filamentous fungi. Biotechnol. Genet. Eng. Rev. **9**: 327-367.

Jefferis, R., J. Lund and J.D. Pound. 1998. IgG-Fc-mediated effector functions: molecular definition of interaction sites for effector ligands and the role of glycosylation. Immunol. Rev. **163**: 59-76.

Jenkins, N., R.B. Parekh and D.C James. 1996. Getting the glycosylation right: implications for the biotechnology industry. Nature Biotechnol. **14**: 975-981.

Jirholt, P., L. Strandberg, B. Jansson, E. Krambovitis, E. Soderlind, C.A. Borrebaeck, R. Carlsson, L. Danielsson and M. Ohlin. 2001. A central core structure in an antibody varoable domain determines antigen specificity. Protein Eng. **14**: 67-74.

Kaetzel, C.S., J.K. Robinson, K.R. Chintalacharuvu, J.P. Vaerman and M.E. Lamm. 1991. The polymeric immunoglobulin receptor (secretory component) mediates transport of immune complexes across epithelial cells: a local defence function for IgA. Proc. Natl. Acad. Sci. USA **88**: 8796-8800.

Kaloff, C.R. and I.G. Haas. 1995. Coordination of immunoglobulin chain folding and immunoglobulin chain assembly is essential for the formation of functional IgG. Immunity **2**: 629-637.

Kawamura, S., N. Saitou and S. Ueda. 1992. Concerted evolution of the primate immunoglobulin alpha-gene through gene conversion. J. Biol. Chem. **267**: 7359-7367.

Khorana, H.G. 1992. Rhodopsin, photoreceptor of the rod cell. An emerging pattern for structure and function. J. Biol. Chem. **267**: 1-4.

Kilian, M., J. Mestecky and M.W. Russell. 1988. Defence mechanisms involving Fc-dependent functions of immunoglobulin A and their subversion by bacterial immunoglobulin A proteases. Microbiol. Rev. **52**: 296-303.

Kilian, M., J. Reinholdt, H. Lomholt, K. Poulsen and E.V. Frandsen. 1996. Biological significance of IgA1 proteases in bacterial colonisation and pathogenesis: critical evaluation of experimental evidence. APMIS **104**: 321-338.

Kim, P.S. and P. Arvan. 1995. Calnexin and BiP act as sequential molecular chaperones during thyroglobulin folding in the endoplasmic reticulum. J. Cell Biol. **128**: 29-38.

Klausner, R.D. and R. Sitia. 1990. Protein degradation in the endoplasmic reticulum. Cell **62**: 611-614.

Knarr, G., M-J. Gething, S. Modrow and J. Buchner. 1995. BiP binding sequences in antibodies. J. Biol. Chem. **270**: 27589-27594.

Knight, K.L., M.L. Vetter and T.R. Malek. 1975. Distribution of covalently bound and non-covalently bound secretory component on subclasses of rabbit secretory IgA. J. Immunol. **115**: 595-598.

Kniskern, P.J., A. Hagopian, P. Burke, L.D. Schulz, D.L. Montgomery, W.M. Hurni. 1994. Characterisation and evaluation of a recombinant hepatitis-B vaccine expressed in yeast defective for N-linked hyperglycosylation. Vaccine **12**: 1021-1025.

Knittler, M.R. and I.G. Haas. 1992. Interaction of BiP with newly synthesised immunoglobulin light chain molecules: cycles of sequential binding and release. EMBO J. **11**: 1573-1581.

Knittler, M.R., S. Dirks and I.G. Haas. 1995. Molecular chaperones involved in protein degradation in the endoplasmic reticulum: quantitative interaction of the heat shock cognate protein BiP with partially folded immunoglobulin light chains that are degraded in the endoplasmic reticulum. Proc. Natl. Acad. Sci. USA **92**: 1764-1768.

Kohler, G. and C. Milstein. 1975. Continuous culture of fused cells secreting antibody of predefined specificity. Nature **256**: 495-497.

Kohler, G., S.C. Howe and C. Milstein. 1976. Fusion between immunoglobulin-secreting and nonsecreting myeloma cell lines. Eur. J. Immunol. **6**: 292-295.

Kohno, K., K. Normington, J. Sambrook, M-J. Gething and K. Mori. 1993. The promoter region of the yeast *KAR2* (BiP) gene contains a regulatory domain that responds to the presence of unfolded proteins in the endoplasmic reticulum. Mol. Cell. Biol. **13**: 877-890.

Kornfeld, R. and S. Kornfeld. 1985. Assembly of asparigine-linked oligosaccharides. Annu. Rev. Biochem. **54**: 631-664.

Koshland, M.E. 1985. The coming of age of the immunoglobulin J chain. Annu. Rev. Immunol. **33**: 425-453.

Kozutsumi, Y., M. Segal, K. Normington, M-J. Gething and J. Sambrook. 1988. The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-related proteins. Nature **332**: 462-464.

Krause, K.H. and M. Michalak. 1997. Calreticulin. Cell **88**: 439-443.

Kroning, H., U. Thiel and S. Ansorge. 1991. Induction of protein disulphide isomerase and immunoglobulins by pokeweed mitogen in human lymphocytes. Hybridoma **10**: 651-657.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**: 680-685.

Laitusis, A.L., M.A. Brostrom and C.O. Brostrom. 1999. The dynamic role of GRP78/BiP in the coordination of mRNA translation with protein processing. J. Biol Chem. **274**: 486–493.

LaMantia, M., T. Miura, H. Tachikawa, H.A. Kaplan, W.J. Lennarz and T. Mizunaga. 1991. Glycosylation site binding protein and protein disulphide isomerase are identical and essential for cell viability in yeast. Proc. Natl. Acad. Sci. USA **88**: 4453–4457.

Lasswell, J., L.E. Rogg, D.C. Nelson, C. Rongey and B. Bartel. 2000. Cloning and characterisation of IAR1, a gene required for auxin conjugate sensitivity in *Arabidopsis*. Plant Cell **12**: 2395-2408.

Leatherbarrow, R.J. and R.A. Dwek. 1984. Binding of complement subcomponent Clq to mouse IgG1, IgG2a and IgG2b; a novel Clq binding assay. Mol. Immunol. **21**: 321-327.

Leborgne-Castel, N., E.P. Jelitto-van Dooren, A.J. Crofts and J. Denecke. 1999. Overexpression of BiP in tobacco alleviates endoplasmic reticulum stress. Plant Cell **11**: 459-469.

Lee, Y-K., J.W. Brewer, R. Hellman and L.M. Hendershot. 1999. BiP and immunoglobulin light chain cooperate to control the folding of heavy chain and ensure the fidelity of immunoglobulin assembly. Mol. Biol. Cell 10: 2209-2219.

Lehner, T., J. Caldwell and R. Smith. 1985. Local passive immunisation by monoclonal antibodies against streptococcal antigen I/II in the prevention of dental caries. Infect. Immun. **50**: 796-799.

Lehner, T., J. Ma, K. Grant and R. Smith. 1986. Local passive immunisation by monoclonal antibodies against *Streptococcus mutans* antigens in the prevention of dental caries. In: T. Lehner and G. Cimasoni (eds.), Borderland between caries and periodontal disease. Medicine and Hygiene, Geneva, pp.347-356.

Leibiger, H., A. Hansen, G. Schoenherr, M. Seifert, D. Wustner, R. Stigler and U. Marx. 1995. Glycosylation analysis of a polyreactive human mononclonal IgG antibody derived from a human-mouse heterohybridoma. Mol. Immunol. **32**: 595-602.

Leibiger, H., D. Wustner, R.D. Stigler and U. Marx. 1999. Variable domain-linked oligosaccharides of a human monoclonal IgG: structure and influence on antigen binding. Biochem. J. **338**: 529-538.

Leno, G.H. and B.E. Ledford. 1990. Reversible ADP-ribosylation of the 78 kDa glucose-regulated protein. FEBS Lett. **276**: 29-33.

Lerouge, P. and L. Faye. 1996. Recent developments in structural analysis of N-glycans from plant glycoproteins. Plant Physiol. Biochem. **34**: 263-271.

Lerouge, P., A-C. Fitchette-Laine, A. Chekkafi, V. Avidgor and L. Faye. 1996. N-linked oligosaccharide processing is not necessary for glycoprotein secretion in plants. Plant J. **10**: 713-719.

Lerouge, P., M. Cabanes-Macheteau, C. Rayon, A.C. Fitchette-Laine, V. Gomord and L. Faye. 1998. N-glycoprotein biosynthesis in plants: recent developments and future trends. Plant Mol. Biol. **38**: 31-48.

Letourneur, O., S. Sechi, J. Willettebrown, M.W. Robertson and J.P. Kinet. 1995. Glycosylation of human truncated Fc-epsilon-R1 α chain is necessary for efficient folding in the endoplasmic reticulum. J. Biol. Chem. **270**: 8249-8256.

Li, X., Y. Wu, D-Z. Zhang, J.W. Gillikin, R.S. Boston, V.R. Franceschi and T.W. Okita. 1993. Rice prolamine protein body biogenesis: a BiP-mediated process. Science **262**: 1054-1056.

Li, C.P. and B.A. Larkins. 1996. Expression of protein disulphide isomerase is elevated in the endosperm of the maize *floury*-2 mutant. Plant Mol. Biol. **30**: 873-882.

Li, X., R.T. Su, H.T. Hsu and H. Sze. 1998. The molecular chaperone calnexin associates with the vacuolar H(+)-ATPase from oat seedlings. Plant Cell **10**: 119-130.

Liljemark, W.F., C.G. Bloomquist and J.C. Ofstehage. 1979. Aggregation and adherence of *Streptococcus sanguis*: role of human salivary immunoglobulin A. Infect. Immun. **26**: 1104-1110.

Lindh, E. 1975. Increased resistance of immunoglobulin A dimers to proteolytic degradation after binding of secretory component. J. Immunol. **114**: 284-286.

Lindquist, S. and E.A. Craig. 1988. The heat shock proteins. Annu. Rev. Genet. **22**: 631-677.

Lipsky, P., E.W. St Clair, D. Furst. 1999. A phase III study of infliximab in patients with active rheumatoid arthritis despite methotrexate. Arthritis Rheumatology **42**: S401.

Logemann, J., J. Schell and L. Willmitzer. 1987. Improved method for the isolation of RNA from plant tissues. Anal. Biochem. **163**: 16-20.

Lomonossoff, G and W.D. Hamilton. 1999. Cowpea mosaic virus-based vaccines. Curr. Top. Microbiol. Immunol. **240**: 177-189.

Loo, T.W. and D.M. Clarke. 1994. Prolonged association of temperature-sensitive mutants of human P-glycoprotein with calnexin during biogenesis. J. Biol. Chem. **269**: 28683-28689.

Lullau, E., S. Heyse, H. Vogel, L. Marison, U. von Stockar, J.-P. Kraehenbuhl and B. Corthesy. 1996. Antigen binding properties of purified immunoglobulin A and reconstituted secretory immunoglobulin A antibodies. J. Biol. Chem. **271**: 16300-16309.

Lupattelli, F. 1997. The rate of phaseolin assembly is controlled by the glycosylation state of its N-linked oligosaccharide chains. Plant Cell **9**: 597-609.

Ma, J.K-C., R. Smith and T. Lehner. 1987. Use of monoclonal antibodies in local passive immunisation to prevent colonisation of human teeth by *Streptococcus mutans*. Infect. Immun. **55**: 1274-1278.

Ma, J.K-C., M. Hunjan, R. Smith and T. Lehner. 1989. Specificity of monoclonal antibodies in local passive immunisation against *Streptococcus mutans*. Clin. Exp. Immunol. **77**: 331-337.

Ma, J.K-C., M. Hunjan, R. Smith, C. Kelly and T. Lehner. 1990. An investigation into the mechanism of protection by local passive immunisation with monoclonal antibodies against *Streptococcus mutans*. Infect. Immun. **58**: 3407-3414.

Ma, J.K-C., T. Lehner, P. Stabila, C.I. Fux and A. Hiatt. 1994. Assembly of monoclonal antibodies with IgG1 and IgA heavy chain domains in transgenic plants. Eur. J. Immunol. **24**: 131-138.

Ma, J.K-C., A. Hiatt, M. Hein, N.D. Vine, F. Wang, P. Stabila, C. van Dolleweerd, K. Mostov and T. Lehner. 1995. Generation and assembly of secretory antibodies in plants. Science **268**: 716-719.

Ma, J.K-C., B.Y. Hikmat, K. Wycoff, N.D. Vine, D. Chargelegue, L. Yu, M.B. Hein and T. Lehner. 1998. Characterisation of a recombinant plant monoclonal secretory antibody and preventive immunotherapy in humans. Nature Medicine **4**: 601-606.

Mach, J.P. 1970. *In vitro* combination of human and bovine free secretory component with IgA of various species. Nature **228**: 1278-1282.

Margolese, L., G.L. Waneck, C.K. Suzuki, E. Degen, R.A. Flavell and D.B. Williams. 1993. Identification of the region on the class I histocompatability molecule that interacts with the molecular chaperone, p88 (calnexin, IP90). J. Biol. Chem. **268**: 17959-17966.

Mariani, M., M. Camaga, L. Tarditi and E. Seccamani. 1991. A new enzymatic method to obtain high-yield F(ab')2 suitable for clinical use from mouse IgG1. Mol. Immunol. **28**: 69-77.

Mattu, T.S., R.J. Pleass, A.C. Willis, M. Kilian, M.R. Wormald, A.C. Lellouch, P.M. Rudd, J.M. Woof and R.A. Dwek. 1998. The glycosylation and structure of human serum IgA1, Fab', and Fc regions and the role of N-glycosylation on Fc alpha receptor interactions. J. Biol. Chem. **273**: 2260-2272.

Mazanec, M.B. C.S. Kaetzel, M.E. Lamm, D. Fletcher and J.G. Nedrud. 1992. Intracellular neutralisation of virus by immunoglobulin A antibodies. Proc. Natl. Acad. Sci. USA **89**: 6901-6905.

McCafferty, J., A.D. Griffiths, G. Winter and D.J. Chiswell. 1990. Phage antibodies: filamentous phage displaying antibody variable domains. Nature **348**: 552-554.

McClellan, A.J., J.B. Endres, J.P. Vogel, D. Palazzi, M.D. Rose and J.L. Brodsky. 1998. Specific molecular chaperone interactions and an ATP-dependent conformational change are required during posttranslational protein translocation into the yeast ER. Mol. Biol. Cell **9**: 3533-3545.

McGhee, J.R. and J. Mestecky. 1990. In defence of mucosal surfaces. Infect. Dis. Clin. North Am. **4**: 315-341.

Meldolesi, J. and T. Pozzan. 1998. The endoplasmic reticulum Ca^{2+} store: a view from the lumen. Trends Biochem. Sci. **23**: 10-14.

Melnick, J., S. Aviel and Y. Argon. 1992. The endoplasmic reticulum stress protein GRP94, in addition to BiP, associates with unassembled immunoglobulin chains. J. Biol. Chem. **267**: 21303-21306.

Melnick, J., J.L. Dul and Y. Argon. 1994. Sequential interaction of the chaperones BiP and GRP94 with immunoglobulin chains in the endoplasmic reticulum. Nature **370**: 373-375.

Melnick, J. and Y. Argon. 1995. Molecular chaperones and the biosynthesis of antigen receptors. Immunol. Today **16**: 243-250.

Menegazzi, P., F. Guzzo, B. Baldan, P. Mariani and S. Treves. 1993. Purification of calreticulin-like protein(s) from spinach leaves. Biochem. Biophys. Res. Commun. **190**: 1130-1135.

Mestecky, J. 1987. The common mucosal immune system and current strategies for induction of immune response in external secretions. J. Clin. Immunol. **7**: 265-276.

Mestecky, J. and J.R.McGhee. 1987. Immunoglobulin A (IgA): molecular and cellular interactions involved in IgA biosynthesis and immune response. Advances in Immunol. **40**: 153-245.

Meunier, L., Y.K. Usherwood, K.T. Chung and L.M. Hendershot. 2002. A subset of chaperones and folding enzymes form multiprotein complexes in endoplasmic reticulum to bind nascent proteins. Mol. Biol. Cell **13**: 4456-4469.

Mian, I.S., A.R. Bradwell and A.J. Olson. 1991. Structure, function and properties of antibody binding sites. J. Mol. Biol. **217**: 133-151.

Michalak, M., R.E. Milner, K. Burns and M. Opas. 1992. Calreticulin. Biochem. J. **285**: 681-692.

Michalak, M., E.F. Corbett, N. Mesaeli, K. Nakamura and M. Opas. 1999. Calreticulin: one protein, one gene, many functions. Biochem. J. **344**: 281-292.

Michalek, S.M., R.L. Gregory, C.C. Harmon, J. Katz, G.J. Richardson, T. Hilton, S.J. Filler and J.R. McGee. 1987. Protection of gnotobiotic rats against dental caries by passive immunisation with bovine milk antibodies to *Streptococcus mutans*. Infect. Immun. **55**: 2341-2347.

Mierendorf, R.C. and D. Pfeffer. 1987. Sequencing of RNA transcripts synthesised *in vitro* from plasmids containing bacteriophage promoters. Meth. Enzymol. **152**: 563-566.

Mirzabekov, T., N. Bannert, M. Farzan, W. Hofmann, P. Kolchinsky, L. Wu, R. Wyatt and J. Sodroski. 1999. Enhanced expression, native purification, and characterisation of CCR-5, a principal HIV-1 coreceptor. J. Biol. Chem. **274**: 28745-28750.

Mitchell, C.G., R. Smith and T. Lehner. 1987. Recognition of carbohydrate and protein epitopes by monnoclonal antibodies to a cell wall antigen from *Streptococcus mutans*. Infect. Immun. **55**: 810-815.

Mizoguchi, A., T. Mizuochi and A. Kobata. 1982. Structures of the carbohydrate moieties of secretory component purified from human milk. J. Biol. Chem. **257**: 9612-9621.

Mogelsvang, S. and D.J. Simpson. 1998. Protein folding and transport from the endoplasmic reticulum to the golgi apparatus in plants. J. Plant Physiol. **153**: 1-15.

Molinari, M. and A. Helenius. 2000. Chaperone selection during glycoprotein translocation into the endoplasmic reticulum. Science **288**: 331-333.

Mostov, K.E., M. Freidlander and G. Blobel. 1984. The receptor for transepithelial transport of IgA and IgM contains multiple immunoglobulin-like domains. Nature **308**: 37-43.

Mostov, K.E. 1994. Transepithelial transport of immunoglobulins. Annu Rev Immunol. **12**: 63-84.

Muller, G. 2000. Towards 3D structures of G protein-coupled receptors: a multidisciplinary approach. Curr. Med. Chem. 7: 861-888.

Munro, S. and H.R.B. Pelham. 1986. An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. Cell **46**: 291-300.

Murray, C.J. and A.D. Lopez. 1994. Global and regional cause-of-death patterns in 1990. Bull. World Health Organ. **72**: 447-480.

Nandan, D., C. Daubenberger, G. Mpimbaza and T.W. Pearson. 1994. A rapid, single-step purification method for immunogenic members of the hsp 70 family: validation and application. J. Immunol. Meth. **176**: 255-263.

Napier, R.M., L.C. Fowke, C. Hawes, M. Lewis and H.R. Pelham. 1992. Immunological evidence that plants use both HDEL and KDEL for targeting proteins to the endoplasmic reticulum. J. Cell Sci. **102**: 261-271.

Nardi, M.C., E. Giacomelli, P. Dainese, A-C. Fitchette-Laine, L. Faye, B. Baldan, L. Navazio and P. Mariani. 1998. *Ginkgo biloba* expresses calreticulin, the major calcium-binding reticuloplasmin in eukaryotic cells. Bot. Acta. **111**: 66-70.

Nauseef, W.M., S.J. McCormick and R.A. Clark. 1995. Calreticulin functions as a molecular chaperone in the biosynthesis of myeloperoxidase. J. Biol. Chem. **270**: 4741-4747.

Neutra, M.R., P. Michetti and J-P. Kraehenbuhl. 1991. Identification and use of protective monoclonal IgA antibodies against viral and bacterial pathogens. Adv. Exp. Med. Biol. **310**: 179-182.

Niles, M.J., L. Matsuuchi and M.E. Koshland. 1995. Polymer IgM assembly and secretion in lymphoid and nonlymphoid cell lines: evidence that J chain is required for pentamer IgM synthesis. Proc. Natl. Acad. Sci. USA **92**: 2884-2888.

Noiva, R. and W.J. Lennarz. 1992. Protein disulphide isomerase: a multifunctional protein resident in the lumen of the endoplasmic reticulum. J. Biol Chem. **267**: 3553-3556.

Noiva, R. 1999. Protein disulphide isomerase: the multifunctional redox chaperone of the endoplasmic reticulum. Seminars Cell Dev. Biol. **10**: 481-493.

Normington, K., K. Kohno, Y. Kozutsumi, M-J. Gething and J. Sambrook. 1989. S. cerevisiae encodes an essential protein homologous in sequence and function to mammalian BiP. Cell **57**: 1223-1236.

Nose, M. and H. Wigzell. 1983. Biological significance of carbohydrate chains on monoclonal antibodies. Proc. Natl. Acad. Sci. USA **80**: 6632-6636.

Nuttall, J., N. Vine, J.L. Hadlington, P. Drake, L. Frigerio and J.K. Ma. 2002. ER-resident chaperone interactions with recombinant antibodies expressed in transgenic plants. Eur. J. Biochem. **269**: 6042-6051.

Odell, J.T., F. Nagy and N.H. Chua. 1985. Identification of DNA sequences required for activity of the cauliflower mosiac virus 35S promoter. Nature **313**: 810-812.

Oi, V,T., S.L. Morrison, L.A. Herzenberg and P. Berg. 1983. Immunoglobulin gene expression in transformed lymphoid cells. Proc. Natl. Acad. Sci. USA **80**: 825-829.

Olden, K., B.A. Bernard, W. Turner and S.L. White. 1982. Effect of interferon on protein glycosylation and comparison with tunicamycin. Nature **300**: 290-292.

Oliver, J.D., F.J. van der Wal, N.J. Bulleid and S. High. 1997. Interaction of the thiol-dependent reductase ERp57 with nasscent glycoproteins. Science **275**: 86-88.

Ostwald, T.J. and D.H. Maclennan. 1974. Isolation of a high affinity calcium-binding protein from sarcoplasmic reticulum. J. Biol. Chem. **249**: 974-979.

Otake, S., Y. Nishihara, M. Makimura, H. Hatta, M. Kim, T. Yamamotto and M. Hirasawa. 1991. Protection of rats against dental caries by passive immunization with hen-egg-yolk antibody (IgY). J. Dent. Res. **70**: 162-166.

Otteken, A. and B. Moss. 1996. Calreticulin interacts with newly synthesised human immunodeficiency virus type I envelope glycoprotein, suggesting a chaperone function similar to that of calnexin. J. Biol. Chem. **271**: 97-103.

Parekh, R.B., R.A. Dwek, B.J. Sutton, D.L. Fernandes, A. Leung, D. Stanworth, T.W. Rademacher, T. Mizuochi, T. Taniguchi, K. Matsuta, F. Takeuchi, Y. Nagano, T. Miyamoto and A. Kobata. 1985. Association of rheumatoid arthritis and primary osteoarthritis with changes in the glycosylation pattern of total serum IgG. Nature **316**: 452-457.

Parekh, R.B., R.A. Dwek, P.M. Rudd, J.R.Thomas, T.W. Rademacher, T. Warren, T.C. Wun, B. Hebert, B. Reitz and M. Palmier. 1989. N-glycosylation and *in vitro* enzymatic activity of human recombinant tissue plasminogen activator expressed in Chinese hamster ovary cells and a murine cell line. Biochem. **28**: 7670-7679.

Park, S.H., S.R. Pinson and R.H. Smith. 1996. T-DNA integration into genomic DNA of rice following Agrobacterium inoculation of isolated shoot apices. Plant Mol. Biol. **32**: 1135-1148.

Parkhouse, R.M. and E. Della Corte. 1973. Biosynthesis of immunoglobulin A (IgA) and immunoglobulin M (IgM). Control of polymerisation by J chain. Biochem. J. **136**: 607-609.

Pedrazzini, E., G. Giovinazzo, R. Bollini, A. Ceriotti and A. Vitale. 1994. Binding of BiP to an assembly-defective protein in plant cells. Plant J. **5**: 103-110.

Pedrazzini, E., G. Giovinazzo, A. Bielli, M. de Virgilio, L. Frigerio, M. Pesca, F. Faoro, R. Bollini, A. Ceriotti and A. Vitale. 1997. Protein quality control along the route to the plant vacuole. Plant Cell 9: 1869-1880.

Pelham, H.R.B. 1986. Speculations on the functions of the major heat shock and glucose-regulated proteins. Cell 46: 959-961.

Percy, J.R., M.E. Percy and R. Baumal. 1976. Assembly of three major subclasses of mouse immunoglobulin G: a theoretical model for covalent assembly *in vivo*. Can. J. Biochem. **54**: 688-698.

Peterson, J.R., A. Ora, P.N. Van and A. Helenius. 1995. Transient, lectin-like association of calreticulin with folding intermediates of cellular and viral glycoproteins. Mol. Biol. Cell 6: 1173-1184.

Phalipon A., A. Cardona, J.P. Kraehenbuhl, L. Edelman, P.J. Sansonetti and B. Corthesy. 2002. Secretory component: a new role in secretory IgA-mediated immune exclusion in vivo. Immunity **17**: 107-115.

Piedimonte, G., K.A. King, N.L. Holmgren, P.J. Bertrand, M.M. Rodriguez and R.L. Hirsch. 2000. A humanised monoclonal antibody against respiratory syncitial virus (palivizumab) inhibits RSV-induced neurogenic-meidated inflammation in rat airways. Pediatr. Res. **47**: 351-356.

Plakidou-Dymock, S., D. Dymock, and R. Hooley. 1998. A higher plant seven-transmembrane receptor that influences sensitivity to cytokinins. Curr. Biol. **8**: 315-324.

Plemper, R.K., S. Bohmler, J. Bordallo, T. Sommer and D. Wolf. 1997. Mutant analysis links the translocon and BiP to retrograde protein transport for degradation. Nature (letts.) **387**: 891-895.

Potter, K.N., Y. Li and J.D. Capra. 1993. Antibody production in the baculovirus system. Int. Rev. Immunol. **10**: 103-112.

Pouyssegur, J., R.P.C. Shiu and I. Pastan. 1977. Induction of two transformation-sensitive membrane polypeptides in normal fibroblasts by a block in glycoprotein synthesis or glucose deprivation. Cell **11**: 941-947.

Puig, A. and H.F. Gilbert. 1994. Protein disulphide isomerase exhibits anti-chaperone activity in the oxidative refolding of lysozyme. J. Biol Chem. **269**: 7764-7771.

Rademacher, T.W., S.W. Homans, R.B. Parekh and R.A. Dwek. 1986. Immunoglobulin G as a glycoprotein. Biochem. Soc. Symp. **51**: 131-148.

Raport, C.J., J. Gosling, V.L. Schweickart, P.W. Gray and I.F. Charo. 1996. Molecular cloning and functional characterisation of a novel human CC chemokine receptor (CCR-5) for RANTES, MIP-1β, and MIP-1α. J. Biol. Chem. **271**: 17161-17166.

Rindisbacher, L., S. Cottet, R. Wittek, J-P. Kraehenbuhl and B. Corthesy. 1995. Production of human secretory component with dimeric IgA binding capacity using viral expression systems. J. Biol. Chem. **270**: 14220-14228.

Riviere, G.R., M.A. Wagoner and I.L. Freeman. 1992. Chronic peroral immunisation of conventional laboratory rats with mutans streptococci leads to stable acquired suppression of salivary antibodies. Oral Microbiol. Immunol. **7**: 137-141.

Roden, L.T., B.J. Miflin and R.B. Freedman. 1982. Protein disulphide isomerase is located in the endoplasmic reticulum of developing wheat endosperm. FEBS Lett **138**: 121-124.

Rogers, S.G., H.J. Klee, R.B. Horsch and R.T. Fraley. 1987. Meth. Enzym. **153**: 253-277.

Roque-Barreira, M.C. and A. Campos-Neto. 1985. Jacalin: an IgA-binding lectin. J. Immunol. **134**: 1740-1743.

Rose, M.D., L.M. Misra and J.P. Vogel. 1989. *KAR2*, a karyogamy gene, is the yeast homolog of the mammalian BiP/GRP78 gene. Cell **57**: 1211-1221.

Royle, L., A. Roos, D.J. Harvey, M.R. Wormald, D. van Gijlswijk-Janssen, R.M. Redwan, I.A. Wilson, M.R. Daha, R.A. Dwek and P.M. Rudd. 2003. Secretory IgA N- and O-glycans provide a link between the innate and adaptive immune system. J. Biol. Chem. **278**: 20140-20153.

Rudd, P.M., R.J. Leatherbarrow, T.W. Rademacher and R.A. Dwek. 1991. Diversification of the IgG molecule by oligosaccharides. Mol. Immunol. **28**: 1369-1378.

Rudd, P.M. and R.A. Dwek. 1997. Glycosylation: heterogeneity and the 3D structure of proteins. Crit. Rev. Biochem. Mol. Biol. **32**: 1-100.

Rudd, P.M. and R.A. Dwek. 1998. The importance of sugars for the function of antibodies. Immunology News 1988 (April): 84-89.

Russell, D.A. 1999. Feasibility of antibody production in plants for human therapeutic use. Curr. Top. Microbiol. Immunol. **240**: 119-138.

Russell, M.W., L.A. Bergmeier, E.D. Zanders and T. Lehner. 1980. Protein antigens of *Streptococcus mutans*: purification and properties of a double antigen and its' protease-resistant component. Infect. Immun. **28**: 486-493.

Russell, M.W., G. Hajishengalis, N.K. Childers and S.M. Michalek. 1999. Secretory immunity in defence against cariogenic mutans streptococci. Caries Res. **33**: 4-15.

Russell-Jones, G.J., P.L. Ey and B.L. Reynolds. 1981. Inhibition of cutaneous anaphylaxis and arthus reactions in the mouse by antigen-specific IgA. Int. Arch. Allergy Appl. Immunol. **66**: 316-325.

Sadasivan, B., P.J. Lehner, B. Ortmann, T. Spies and P. Cresswell. 1996. Roles for calreticulin and a novel glycoprotein, tapasin, in the interaction of MHC class I molecules with TAP. Immunity **5**: 103-114.

Saltzman, W.M., M.L. Radomsky, K.J. Whaley and R.A. Cone. 1994. Antibody diffusion in human cervical mucus. Biophys J. **66**: 508-515.

Sambrook, J., E.F. Fritsch and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. 2nd Edition. Cold Spring Harbor Laboratory Press.

Samson, M., O. Labbe, C. Mollereau, G. Vassart and M. Parmentier. 1996. Molecular cloning and functional expression of a new human CC-chemokine receptor gene. Biochem. **35**: 3362-3367.

Sanders, S.L., K.M. Whitfield, J.P. Vogel, M.D. Rose and R. Schekman. 1992. Sec61p and BiP directly facilitate polypeptide translocation into the ER. Cell **69**: 353-365.

Schaerer, E., M.R. Neutra and J-P. Kraehenbuhl. 1991. Molecular and cellular mechanisms involved in transepithelial transport. J. Membr. Biol. **123**: 93-103.

Schneiderman, R.D., W.C. Hanly and K.L. Knight. 1989. Expression of 12 rabbit IgA C alpha genes as chimeric rabbit-mouse IgA antibodies. Proc. Natl. Acad. Sci. USA **86**: 7561-7565.

Schoneberg T., G. Schultz and T. Gudermann. 1999. Structural basis of G protein-coupled receptor function. Mol. Cell Endocrinol. **151**: 181-193.

Secher, D.S., C. Milstein and K. Adetugbo. 1977. Somatic mutants and antibody diversity. Immnol. Rev. **36**: 51-72.

Shamu, C.E., J.S. Cox and P. Walter. 1994. The unfolded-protein-response pathway in yeast. Trends Cell Biol. **4**: 56-60.

Shimoni, Y., X. Zlu, H. Levanony, G. Segal and G. Galili. 1995. Purification, characterisation, and intracellular localisation of glycosylated protein disulphide isomerase from wheat grains. Plant Physiol. **108**: 327-335.

Shin, S.U., A. Wright and S.L. Morrison. 1993. Hybrid antibodies. Int. Rev. Immunol. **10**: 177-186.

Shorrosh, B.S. and R.A. Dixon. 1991. Molecular cloning of a putative plant endomembrane protein resembling vertebrate protein disulphide isomerase and a phosphatidylinositol-specific phospholipase C. Proc. Natl. Acad. Sci. USA **88**: 10941-10945.

Shusta, E.V., R.T. Raines, A. Pluckthun and K.D. Wittrup. 1998. Increasing the secretory capacity of *Saccharomyces cerevisiae* for production of single-chain antibody fragments. Nat. Biotechnol. **16**: 773-777.

Simmons, L.C., D. Reilly, L. Klimowski, T. Shantha Raju, G. Meng, P. Sims, K. Hong, R.L. Shields, L.A. Damico, P. Rancatore and D.G. Yansura. 2003. Expression of full-length immunoglobulins in *Escherichia coli*: rapid and efficient production of aglycosylated antibodies. J. Immunol. Meth. **263**: 133-147.

Smith, R. and T. Lehner. 1989. Characterisation of monoclonal antibodies to common protein epitopes on the cell surface of *Streptococcus mutans* and *Streptococcus sobrinus*. Oral Microbiol. Immunol. **4**: 153-158.

Smith, R.I. and S.L. Morrison. 1994. Recombinant polymeric IgG: an approach to engineering more potent antibodies. Biotechnology **12**: 683-688.

Song, J.L. and C.C. Wang. 1995. Chaperone-like activity of protein disulphide-isomerase in the refolding of rhodenase. Eur. J. Biochem. **231**: 312-316.

Sonnichsen, B., J. Fullekrug, P. Van Nguyen, W. Diekmann, D.G. Robinson and G. Mieskes. 1994. Retention and retrieval: both mechanisms cooperate to maintain calreticulin in the endoplasmic reticulum. J. Cell Sci. **107**: 2705-2717.

Sorensen, V., I.B. Rasmussen, V. Sundvold, T.E. Michaelsen and I. Sandlie. 2000. Structural requirements for incorporation of J chain into human IgM and IgA. Int. Immunol. 12: 19-27.

Sousa, M.C., M.A. Ferrero-Garcia and A.J. Parodi. 1992. Recognition of the oligosaccharide and protein moieties of glycoproteins by the UDP-Glc:glycoprotein glucosyltransferase. Biochem. **31**: 97-105.

Spieker-Polet, H., P.C. Yam and K.L. Knight. 1993. Differential expression of 13 IgA-heavy chains in rabbit lymphoid tissues. J. Immunol. **150**: 5457-5465.

Spiro, R.G., Q. Zhu, V. Bhoyroo and H.D. Soling. 1996. Definition of the lectin-like properties of the molecular chaperone, calreticulin, and demonstration of its copurification with endomannosidase from rat liver Golgi. J. Biol. Chem. **271**: 11588-11594.

Stanasila, L., F. Pattus and D. Massotte. 1998. Heterologous expression of G-protein-coupled receptors: human opioid receptors under scrutiny. Biochimie **80**: 563-571.

Steward, M.W. and D. Chargelegue. 1997. Overview: Measurement and biological significance of antibody affinity and reactivity. In: D.M. Weir, L.A. Herzenberg and C.C. Blackwell (eds.). Handbook of experimental immunology, Blackwell Scientific, Oxford, pp. 38.1.

Stokes, C.R., J.F. Soothill and M.W. Turner. 1975. Immune exclusion is a function of IgA. Nature **255**: 745-746.

Sturm, A., J.A. Van Kuik, J.F. Vliegenthart and M.J. Chrispeels. 1987. Structure, position, and biosynthesis of the high mannose and the complex oligosaccharide side chains of the bean storage protein phaseolin. J. Biol. Chem. **262**: 13392-13403.

Tao, M.H. and S.L. Morrison. 1989. Studies of aglycosylated chimeric mouse-human IgG: role of carbohydrate in the structure and effector functions mediated by the human IgG constant region. J. Immunol. **143**: 2595- 2601

Tate, C.G. and R. Grisshammer. 1996. Heterologous expression of G-protein-coupled receptors. Trends Biotechnol. **14**: 426-30.

Tatu, U. and A. Helenius. 1997. Interactions between newly synthesised glycoproteins, calnexin and a network of resident chaperones in the endoplasmic retiulum. J. Cell Biol. **136**: 555-565.

Taussig, M.J. 1988. Molecular genetics of immunoglobulins. Immunol. Suppl. **1**: 7-15.

Tavladoraki, P., E. Benvenuto, S. Trinca, D. De Martinis, A. Cattaneo and P. Galeffi. 1993. Transgenic plants expressing a functional single-chain Fv antibody are specifically protected from virus attack. Nature **366**: 469-472.

Taylor, H.P and N.J. Dimmock. 1985. Mechanisms of neutralisation of influenza virus by secretory IgA is different from that of monomeric IgA or IgG. J. Exp. Med. **161**: 198-209.

Taylor, A.K. and R. Wall. 1988. Selective removal of a heavy-chain glycosylation sites causes immunoglobulin A degradation and reduced secretion. Mol. Cell. Biol. **8**: 4197-4203.

Terskikh, A., S. Couty, A. Pelegrin, N. Hardman, W. Hunziker and J.P. Mach. 1994. Dimeric recombinant IgA directed against carcino-embryonic antigen, a novel tool for carcinoma localisation. Mol. Immunol. **31**: 1313-1319.

Ting, J., S.K. Wooden, R. Kriz, K. Kelleher, R.J. Kaufman and A.S. Lee. 1987. The nucleotide sequence encoding the hamster 78-kDa glucose-regulated protein (GRP78) and its conservation between hamster and rat. Gene **55**: 147-152.

Tinland, B. 1996. The integration of T-DNA into plant genomes. Trends Plant Sci. **1**: 178-184.

Torano, A., Y. Tsuzukida, Y.S. Liu and F.W. Putnam. 1977. Location and structural significance of the oligosaccharides in human IgA1 and IgA2 immunoglobulins. Proc. Natl. Acad. Sci. USA **74**: 2301-2305.

Trill, J.J., A.R. Shatzman and S. Ganguly. 1995. Production of monoclonal antibodies in COS and CHO cells. Curr. Opin. Biotechnol. **6**: 553-560.

Tyler, B.M., A.F. Cowman, S.D. Gerondakis, J.M. Adams and O. Bernard. 1982. mRNA for surface immunoglobulin gamma chains encodes a highly conserved transmembrane sequence and a 28-residue intracellular domain. Proc. Natl. Acad. Sci. USA **79**: 2008-2012.

Underdown, B.J. and K.J.Dorrington. 1974. Studies on the structural and conformational basis for the relative resistance of serum and secretory immunoglobulin A to proteolysis. J.Immunol. **112**: 949-959.

Van Engelen, F.A., A. Schouten, J.W. Molthoff, J. Roosien, J. Salinas, W.G. Dirkse, A. Schots, J. Bakker, F.J. Gommers and M.A. Jongsma. 1994. Coordinate expression of antibody subunit genes yields high levels of functional antibodies in roots of transgenic tobacco. Plant Mol. Biol. **26**: 1701-1710.

Vanhove, M., Y.K. Usherwood and L.M. Hendershot. 2001. Unassembled Ig heavy chains do not cycle from BiP in vivo but require light chains to trigger their release. Immunity **15**: 105-114.

Van Leeuwen, JE.M. and K.P. Kearse. 1996. The related molecular chaperones calnexin and calreticulin differentially associate with nascent T cell antigen receptor proteins within the endoplasmic reticulum. J. Biol. Chem. **271**: 25345-25349.

Veazey, R.S., R.J. Shattock, M. Pope, J.C. Kirijan, J. Jones, Q. Hu, T. Ketas, P.A. Marx, P.J. Klasse, D.R. Burton and J.P. Moore. 2003. Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120. Nat. Med. 9: 343-346.

Vine, N.D., P. Drake, A. Hiatt and J.K-C. Ma. 2001. Assembly and plasma membrane targeting of recombinant immunoglobulin chains in plants with a murine immunoglobulin transmembrane sequence. Plant Mol. Biol. 45: 159-167.

Vitale, A., A. Ceriotti and J. Denecke. 1993. The role of the endoplasmic reticulum in protein synthesis, modification and intracellular transport. J. Exp. Bot. 44: 1417-1444.

Vitale, A.. 2001. Uncovering secretory secrets: inhibition of endoplasmic reticulum (ER) glucosidases suggests a crictical role for ER quality control in plant growth and development. Plant Cell **13**: 1260-1262.

Vogel, J.P., L.M. Misra and M.D. Rose. 1990. Loss of BiP/GRP78 function blocks translocation of secretory proteins in yeast. J. Cell Biol. **110**: 1885-1895.

Von Heijne, G. 1984. How signal sequences maintain cleavage specificity. J. Mol. Biol. **173**: 243-251.

Wall, R. and M. Kuehl. 1983. Biosynthesis and regulation of immunoglobulins. Annu. Rev. Immunol. **1**: 393-422.

Walther-Larsen, H., J. Brandt, D.B. Collinge and H. Thordal-Christensen. 1993. A pathogen-induced gene of barley encodes a HSP90 homologue showing striking similarity to vertebrate forms resident in the endoplamic reticulum. Plant Mol. Biol. **21**: 1097-1108.

Ward, M., C. Lin, D.C. Victoria, B.P. Fox, J.A. Fox, D.L. Wong, H.J. Meerman, J.P. Pucci, R.B. Fong, M.H. Heng, N. Tsurushita, G. Gieswein, M. Park and H. Wang. Characterisation of humanised antibodies secreted by *Aspergillus niger*. Appl. Environ. Microbiol. - in press.

Ware, F.E., A. Vassilakos, P.A. Peterson, M.R. Jackson, M.A. Lehrman and D.B. Williams. 1995. The molecular chaperone calnexin binds Glc1Man9GlcNAc2 oligosaccharide as an initial step in recognising unfolded glycoproteins. J. Biol. Chem. **270**: 4697-46704.

Weinberg, A., S. Krisanaprakornkit and B.A. Dale. 1998. Epithelial antimicrobial peptides: review and significance for oral applications. Crit. Revs. Oral Biol. Med. **9**: 399-414.

Whaley, K.J., L. Zeitlin and R.A. Barratt. 1994. Passive immunisation of the vagina protects mice against vaginal transmission of genital herpes infections. J. Infect. Dis. **169**: 647-649.

Wiersma, E.J., F. Chen, R. Bazin, C. Collins, R.H. Painter, R. Lemieux and M.J. Shulman. 1997. Analysis of IgM structures involved in J chain incorporation. J. Immunol. **158**: 1719-1726.

Winner III, L., J. Mack, R. Weltzin, J. Mekalanos, J-P. Krachenbuhl and M. Neutra. 1991. New model for analysis of mucosal immunity: intestinal secretion of specific monoclonal immunoglobulin A from hybridoma tumours protects against *Vibrio cholerae* infection. Infect. Immun. **59**: 977-982.

Winter, G., A.D. Griffiths, R.E. Hawkins and H.R. Hoogenboom. 1994. Making antibodies by phage display technology. Annu. Rev. Immunol. **12**: 433-455.

Wold, A.E., C. Motas, C. Svanborg, L.A. Hanson and J. Mestecky. 1995. Characterisation of IgA1, IgA2 and secretory IgA carbohydrate chains using plant lectins. Adv. Exp. Med. Biol. **371A**: 585-589.

Wood, C.R., M.A. Boss, J.H. Kenten, J.E. Calvert, N.A. Roberts and J.S. Emtage. 1985. The synthesis and *in vivo* assembly of functional antibodies in yeast. Nature **314**: 446-449.

Wormald, M.R., E.W. Wooten, R. Bazzo, C.J. Edge, A. Feinstein, T.W. Rademacher and R.A. Dwek. 1991. The conformational effects of N-glycosylation on the tail-piece from serum IgM. Eur. J. Biochem. **198**: 131-139.

Wormald, M.R., P.M. Rudd, D.J. Harvey, S.C. Chang, I.G. Scragg and R.A. Dwek. 1997. Variations in oligosaccharide-protein interactions in immunoglobulin G determine the site-specific glycosylation profiles and modulate the dynamic motion of the Fc oligosaccharides. Biochem. **36**: 1370-1380.

Wright, A. and S.L. Morrison. 1997. Effect of glycosylation in antibody function: implications for genetic engineering. Trends Biotechnol. **15**: 26-32.

Xu, A., A.R. Bellamy and J.A. Taylor. 1998. BiP (GRP78) and endoplasmin (GRP94) are induced following rotavirus infection and bind transiently to an endoplasmic reticulum-localised virion component/ J. Virol. **72**: 9865-9872.

Xu, Z.J., K. Ueda, K. Masuda, M. Ono and M. Inoue. 2002. Molecular characterisation of a novel protein disulphide isomerase in carrot. Gene **284**: 225-231.

Yamawaki-Kataoka, Y., S. Nakai, T. Miyata and T. Honjo. 1982. Nucleotide sequences of gene segments encoding membrane domains of immunoglobulin gamma chains. Proc. Natl. Acad. Sci. USA 79: 2623-2627.

Yoo, M., M.J. Coloma, K.R. Trinh, T.Q. Nguyen, L-U.C. Vuong, S.L. Morrison and K.R. Chintalacharuvu. 1999. Structural requirements for polymeric immunoglobulin assembly and association with J chain. J. Biol. Chem. **274**: 33771-33777.

Yusibov, V.M., I.R. Steck, V. Gupta, and S.B. Gelvin. 1994. Association of single-stranded transferred DNA from *Agrobacterium tumefaciens* with tobacco cells. Proc. Natl. Acad. Sci. USA **91**: 2994-2998.

Zala, C.A., M. Salas-Prato, W.T. Yan, B. Banjo and J.F. Perdue. 1980. In cultured chick embryo fibroblasts the hexose transport components are not the 75 000 and 95 000 dalton polypeptides synthesised following glucose deprivation. Can. J. Biochem. **58**: 1179-1188.

Zambryski, P., J. Tempe and J. Schell. 1989. Transfer and function of T-DNA genes from agrobacterium Ti and Ri plasmids in plants. Cell **56**: 193-201.

Zeitlin, L., S.S. Olmsted, T.R. Moench, M. Sung Co, B.J. Martinell, V.M. Paradkar, D.R. Russell, C. Queen, R.A. Cone and K.J. Whaley. 1998. A humanised monoclonal antibody produced in transgenic plants for immunoprotection of the vagina against genital herpes. Nature Biotechnol. **16**: 1361-1364.

Zeitlin, L., R.A. Cone and K.J. Whaley. 1999. Using monoclonal antibodies to prevent mucosal transmission of epidemic infectious diseases. Emerg. Infec. Dis. **5**: 54-64.

Zhao, J., L. Ma, Y.L. Wu, P. Wang, W. Hu and G. Pei. 1998. Chemokine receptor CCR-5 functionally couples to inhibitory G proteins and undergoes desensitisation. J. Cell Biochem. **71**: 36-45.

Zimmermann, R., M. Sagstetter, M.J. Lewis and H.R.B. Pelham. 1988. Seventy-kilodalton heat shock proteins and an additional component from reticulocyte lysate stimulate import of M13 procoat protein into microsomes. EMBO J. **7**: 2875-2880.

Zimmerman, P.A., A. Buckler-White, G. Alkhatib, T. Spalding, J. Kubofcik, C. Combadiere, D. Weissman, O. Cohen, A. Rubbert, G. Lam, M. Vaccarezza, P.E. Kennedy, V. Kumaraswami, J.V. Giorgi, R. Detels, J. Hunter, M. Chopek, E.A. Berger, A.S. Fauci, T.B. Nutman and P.M. Murphy. 1997. Inherited resistance to HIV-1 conferred by an inactivating mutation in CC chemokine receptor 5: studies in populations with contradicting clinical phenotypes, defined racial background, and quantified risk. Mol. Med. **3**: 23-36.

Zu Putlitz, J.Z., W.L. Kubasek, M. Duchene, M. Marget, B-U. von Specht and H. Domdey. 1990. Antibody production in baculovirus-infected cells. Biotechnol. **8**: 651-654.

Zylber, L.J. and H.V. Jordan. 1982. Development of a selective medium for detection and enumeration of *Actinomyces viscosus* and *Actinomyces naeslundii* in dental plaque. J. Clin. Microbiol. **15**: 253-259.

APPENDIX

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APPENDIX

All chemicals were ordered from SigmaAldrich, UK, unless stated otherwise.

1 BUFFERS AND SOLUTIONS

Alkaline phosphatase substrate buffer: 100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5, 300 mg ml^{-1} nitroblue tetrazolium (NBT), and 150 mg ml^{-1} 5-bromo-4-chloro-3-indolyl phosphate (BCIP). NBT solution and BCIP, both 50 mg ml^{-1}, stored at -20 ^{0}C.

Ampicillin, 1000 X: 100 mg ml^{-1} ampicillin was dissolved in distilled water, sterilised by filtration and stored at - 20 ^{0}C.

Complete™ protease inhibitor mix (Roche Diagnostics, UK): one tablet was dissolved in 2 ml H_2O to make a 12.5 X stock. 1/12.5 volume of this stock was added to protoplast homogenisation buffer just before use.

DEPC-treated water : 2 ml diethylpyrocarbonate (DEPC) was added to 2 L distilled water, left at 37 °C overnight and autoclaved prior to use.

Deionised formamide: 5 g of mixed-bed resin [BioRad - AG501-X8(D)] was added to 100 ml of formamide and gently stirred for 30 minutes, filtered and stored at -20 °C.

DNA loading buffer, 6 X: 0.25 % bromophenol blue, 0.25 % xylene blue, 40 % sucrose.

Enzyme digestion mix, 10 X: 4 % Cellulase (Calbiochem, UK), 2 % Pectinase (Calbiochem, UK), dissolved in K3 medium, stirred vigorously for 20 minutes, collected and centrifuged at 14 000 g for 15 minutes at 4°C to precipitate insoluble material. The mixture was filter sterilised, aliquoted and stored at –20 °C, avoiding more than two freeze-thaw cycles.

Formaldehyde gel running buffer, 5 X: 0.1 M MOPS, pH 7.0, 40 mM sodium acetate, and 5 mM EDTA, pH 8.

IPTG: a solution of 1 M Isopropyl-β-D thiogalactopyranoside (IPTG) was made in distilled water, filter sterilised, aliquoted and stored at –20 °C.

K3 medium: 3.78g Gamborg's B5 basal medium with minimal organics, 750 mg $CaCl_2.2H_2O$, 250 mg NH_4NO_3, 136.2 g sucrose, 250 mg xylose, 1mg 6-benzylaminopurine, 1mg α-napthaleneacetic acid in 1 L distilled water. A stock solution of 5 mg ml^{-1} 6-benzylaminopurine in 1M NaOH was adjusted to pH 5.5 with a few drops of 1 M KOH, filter sterilised , and stored at –20 °C.

Luria Bertani (LB) broth: 10 g NaCl, 10 g bactotryptone, 5 g yeast extract in 1 L distilled water, adjust to pH 7.4, and autoclave.

Luria Bertani (LB) agar medium: 1 L of LB broth and 15 g bacteriological agar.

MOPS buffer, 10 X: 0.2 M 3-[N-morpholino]propanesulfonic acid) [MOPS], 10 mM EDTA, 50 mM sodium acetate. The pH was adjusted to 7 with NaOH, and then autoclaved prior to use.

NET buffer, 1 X: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA , 0.1 % Nonidet P-40, and 0.02 % sodium azide.

NET-gelatin buffer, 1 X: as NET buffer supplemented with 0.025 % gelatin. A 2 % stock of gelatin in water was autoclaved, and sodium azide added to 0.02 %, then stored at 4 °C. The inclusion of gelatin in the buffer is necessary to reduce non-specific co-precipitation of contaminants.

PBS, 10 X: 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 800 ml distilled water, adjust to 1 L, pH7.4.

PBST, 1 X: 1 X PBS (pH 7.4) containing 0.5 % Tween 20.

Prehybridisation mixture: 50 % deionised formamide, 5 X SSPE, and 5 % SDS. The components were mixed in this order, and the mixture kept at 60 °C to prevent precipitation of SDS.

10 % Protein A-sepharose suspension: Protein A-sepharose (AmershamPharmacia Biotech, UK) was swollen in NET buffer for at least 3 hours with occasional agitation, in a 50 ml tube. Once the beads had sedimented, the supernatant was carefully removed. Tris-HCl (1M, pH 7.5) was added to the beads, and allowed to stand for 1 hour with occasional agitation. The supernatant was removed, and the beads washed once with NET buffer. The beads were allowed to sediment, the supernatant was removed, and 9 bead volumes of NET buffer were added. The suspension was stored at 4 °C.

Protoplast homogenisation buffer: 200 mM Tris-HCl, pH 8, 300 mM NaCl, 1 % Triton X-100, and 1 mM EDTA, stored at –20 °C in aliquots, and 1 % (v/v) Nonidet P40 (NP40) was added when stated.

RNA sample loading buffer, 5 X: 50 % glycerol, 1 mM EDTA, pH 8, 0.25 % bromophenol blue and 0.25 % xylene cyanol.

SDS-PAGE resolving gel - 10 %: 10 ml of 30 % Acrylamide/bis (19:1) [Scotlab, UK], 7.5 ml of 1.5M Tris-HCl (pH 8.8), 0.3 ml 10 % (w/v) SDS, 12 ml distilled water, 225 µl 10 % (w/v) ammonium peroxodisulphate (APS), 15 µl NNN'N'-tetramethyethylene-diamine (TEMED).

SDS-PAGE resolving gel - 4 %: 3.9 ml of 30 % Acrylamide/bis (19:1) [Scotlab, UK], 7.5 ml of 1.5 M Tris-HCl (pH 8.8), 0.3 ml 10 % (w/v) SDS, 17.85 ml distilled water, 300 µl 10 % (w/v) APS, 30 µl TEMED.

SDS-PAGE stacking gel - 5 %: 5.1 ml 30 % Acrylamide/bis (19:1) [Scotlab, UK], 3.75 ml 0.5 M Tris-HCl (pH 6.8), 300 µl 10 % (w/v) SDS, 20.4 ml distilled water, 300 µl 10 % (w/v) APS and 30 µl TEMED.

SDS-PAGE sample loading buffer, 2 X: 125 mM Tris-HCl (pH 6.8), 4 % SDS, 0.2 % bromophenol blue, 20 % glycerol, 200 mM dithiothreitol, 2 % β-mercaptoethanol.

SDS-PAGE running buffer, 5 X: 15.1 g Tris base, 72 g glycine, 900 ml distilled water, 50 ml 10 %(w/v) SDS, adjust volume to 1 L.

Sequencing gel: 34.5 g Urea , 11.25 ml Acrylamide/bis (37.5:1) [Scotlab, UK], 15 ml TBE (5 X), adjust volume with distilled water to 75 ml and add 105 µl TEMED, 255 µl 10 % (w/v) APS. Pour gel using a syringe, avoiding air bubbles. The glass plates must be degreased using alcohol prior to use. The gel is light sensitive.

Sequencing fixing solution: 5 % glacial acetic acid, 5 % methanol, adjust the volume with distilled water to 2 L.

SOC medium: To 950 ml distilled water, add 20 g tryptone, 5 g yeast extract, 0.5 g NaCl, dissolve, and add 250 mM KCl to pH 7.0. Adjust volume to 1 L, autoclave and when cold add 20 mM glucose.

SSC, 20 X: 3 M NaCl, 0.3 M $C_6H_5Na_3O_7 .2H_2O$, pH 7 adjusted with 1M HCl, sterilised by autoclaving.

SSPE, 20 X: 3.6 M NaCl , 0.2 M Na_2HPO_4, and 20 mM EDTA, pH 6.5, sterilised by autoclaving.

TAE buffer, 50 X: 242 g Tris base, 57.1 ml glacial acetic acid, 100 ml EDTA 0.5 M (pH 8.0), adjust volume to 1 L.

TBE buffer, 5 X: 54 g Tris base, 27.5 g boric acid, 20 ml EDTA 0.5M (pH 8.0), adjust volume to 1 L with distilled water.

TBS, 10 X: 88 g NaCl, 12.12 g Tris base, pH 8.0, adjust volume to 1 L with distilled water.

TE buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0).

Tris-HCl 1M, pH 8: 121.1g of Tris base was dissolved in 800 ml H_2O, and 42 ml HCl added. The solution was sterilised by autoclaving.

Tris-HCl 1M, pH 7.4: 121.1 g of Tris base was dissolved in 800 ml H_2O, and 70 ml HCl added. The solution was sterilised by autoclaving.

Western blotting transfer buffer: 15 g Tris base, 72 g glycine, 1 L methanol, adjust volume to 5 L with distilled water.

W5 buffer: 9 g NaCl, 0.37 g KCl, 18.37 g $CaCl_2.2H_2O$, 0.9 g glucose per litre.

X-Gal: a solution of 40 mg ml^{-1} 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was made in dimethyl-formamide. The tube was stored in the dark at –20 °C

2 OLIGONUCLEOTIDES

Oligonucleotides were purchased from Oswel, UK.

J chain oligonucleotides (21-mers);

3' primer	5'	TAT GAA TTC CTA GTC AGG GTA	3'
5' primer	5'	TAT CTC GAG ATG AAG ACC CAC	3'

γ1 heavy chain oligonucleotides (27-mers);

3' primer	5'	CTT AAG CTT ACA ATC CCT GGG CAC AAT	3'
5' primer	5'	CTT CTC GAG TCA GGA CCT GAC CTG GTG	3'

Constitutive chloroplast ribonucleoprotein (rubisco) oligonucleotides (18-mers);

3' primer	5'	AGA TAC GGG AGC TTT CTG	3'
5' primer	5'	GGG TGC ATG TTA GAT GGT	3'

CCR-5 oligonucleotides;

3' primer	5'	TGC TCT AGA CTA CAA GCC CAC AGA TAT TTC	3'
5' primer	5'	CAT GCC ATG GAT TAT CAA GTG TCA	3'

β-actin oligonucleotides (26-mers);

3' primer	5'	CCT AGA AGC ATT TGC GGT GCA CGA TG	3'
5' primer	5'	TCA TGA AGT GTG ACG TTG ACA TCC GT	3'

T3 sequencing primer (17-mer);

5'		ATT AAC CCT CAC TAA AG	3'

T7 sequencing primer (17-mer);

5'		TTA ATA CGA CTC ACT AT	3'

3 MOLECULAR BIOLOGY TECHNIQUES

3.1 POLYMERASE CHAIN REACTION (PCR)

Approximately 5 ng of template DNA was amplified in 100 µl reaction mix comprising 50 pmol oligonucleotide primers, 200 mM dNTP, 10µl 10 X PCR buffer [100mM Tris-HCl (pH 9.0), 15 mM $MgCl_2$, 500 mM KCl] with 2.5 U Taq polymerase. The mixture was overlaid with 20 - 30 µl of mineral oil.

Amplification was for 30 cyles: 30 seconds at 95 °C for denaturing, 30 seconds at 55 °C for annealing and 1 minute at 72 °C for extension. This was followed by a final extension at 72 °C for 10 minutes. Aliquots of the reaction mix were analysed by agarose gel electrophoresis. Samples were either used immediately for cloning or frozen at - 20°C.

3.2 GENERAL LIGATION

Purified DNA was ligated at a molar ratio of insert:vector (2:1). The reaction comprised; the insert, vector, 10 U of T4 DNA ligase, 1X ligation buffer [20 mM Tris-HCl, 1mM EDTA, 5 mM DTT, 60 mM KCl, 50 % (v/v) glycerol, (pH 7.5) AmershamPharmacia Biotech, UK], in a total volume of 20 ml. Ligations were incubated at 4 °C for 16 hours.

3.3 DESALTING LIGATION SAMPLES

50 - 100 µl of glass beads (425 - 600 microns, SigmaAldrich, UK) were placed in a pierced centrifuge tube followed by 250 µl of Sepharose CL-6B (AmershamPharmacia Biotech, UK). The tube was inserted into a second microfuge tube and centrifuged at 1500 x g for 1 minute. The second tube was discarded and replaced with a fresh one. The sample to be de-salted was applied to the Sepharose and nucleic acid was recovered by centrifugation as before.

3.4 RESTRICTION ENZYME DIGESTS

For analysis or isolation of inserts, isolated plasmid was incubated with 5 – 10 Units of restriction enzymes (New England Biolab Inc. UK) per µg of DNA. The digests were then

analysed by agarose gel electrophoresis. When required, digestion reactions were stopped by heat inactivation at 70 °C for 10 minutes.

3.5 CLEANING PLASMID DNA SAMPLES

To prepare plasmid DNA for sequence analysis, pellets were dissolves in 200 µl of sterile distilled water. An equal volume of phenol : chloroform : isoamyl alcohol (24 : 24 : 1, v/v/v) [SigmaAldrich, UK] was added to the sample, which was vortexed and centrifuged for 5 minutes at 15 000 g. The aqueous phase was transferred to a clean tube and 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.2 volumes of ethanol 100 % was added and the sample kept on ice for 5 minutes. DNA was recovered by centrifugation for 20 minutes at 15 000 g. The pellet was washed in 80 % ethanol (1 ml), kept on ice for 5 minutes and then centrifuged for 10 minutes at 15 000 g. The pellet was air-dried before being dissolved in 20 µl of TE buffer. Samples were stored at – 20 °C and aliquots were analysed by agarose electrophoresis.

3.6 DNA SEQUENCING

Sequencing reactions were performed using the SequenaseTM Version 2.0 DNA sequencing kit (USB Amersham Life Sciences, USA). Double stranded DNA (ds DNA), either plasmid or PCR product (1-5 µg in 5 µl) was denatured by incubation with 2 µl of NaOH (2N) for 10 minutes at room temperature. DNA was recovered by spin column chromatography (refer to Appendix section 3.3) into a tube containing 1 ml of primer at 5 pmolμl^{-1} and 2 µl of 5X buffer (200 mM Tis-HCl (pH 7.5), 100 mM $MgCl_2$, 250 mM NaCl). Labelling mix (5.5. µl) comprising for each reaction of 1 µl of 0.1 M DTT, 2 µl of labelling mix buffer (7.5 µM dGTP, 7.5 µM dCTP, 7.5 µM dTTP; diluted 1/5 in water), 0.5 µl (0.185 MBq) of [^{35}S]-dATP, and 2 µl of T7 DNA sequenase (13 Uμl^{-1}) diluted 1/8 in diluting buffer (10 mM Tris-HCl (pH 7.5), 5 mM DTT, 0.5 mg ml^{-1} BSA) to the DNA. 3.5 µl of the mix was then added to four tubes containing 2.5 µl of ddNTPS (A-C-G-T). The samples were incubated at 37 °C for 5 minutes. Reactions were stopped by adding 4 µl of stop buffer (95 % formamide, 20 mM EDTA, 0.05 % bromophenol blue, 0.05 % xylene cyanol FF). Samples were analysed immediately or stored at –20 °C.

Samples to be analysed were heated for 2 minutes at 90 °C, immediately put on ice and aliquots (3 µl) were applied to a sequencing gel. The gel was run with parameters set at 2000 V, 50 – 60 mA and 50 – 60 W. Electrophoresis was for 30 minutes to 6 hours depending on the distance from the primers that had to be sequenced. Gels were then transferred to a fixing solution for 30 minutes before being dried on a heat vacuum device and exposed to Kodak Biomax MR-1 X-ray film. The film was developed using the Fuji RGII automated sequencer and the sequence was read manually.

3.7 GENERAL MATERIALS

Enzymes: Taq DNA polymerase enzyme was from AmershamPharmacia Biotech, UK. RNAse and Dnase, were from New England Biolabs Inc., UK.

Molecular mass markers: DNA size markers were purchased from Gibco BRL, UK.

Radioisotopes: [^{35}S]-dATP was purchased from ICN Biomedicals, UK.

4 MURINE AND PLANT J CHAIN SEQUENCES

The plant J chain PCR product was sequenced and found to be identical to the murine J chain (Matsuuchi *et al.*, 1986), except for a single codon (highlighted in Figure 1a below) described in Chapter 5, section 5.3.

```
ACCAGATCTATGAAGACCCACCTGCTTCTCTGGGGAGTCCTCGCCATTTTTGTTAAGGTT    60
          M  K  T  H  L  L  L  W  G  V  L  A  I  F  V  K  V      20

GTCGACGACGAAGCGACCATTCTTGCTGACAACAAATGCATGTGTACCCGAGTTACCTCT   120
V  D  D  E  A  T  I  L  A  D  N  K  C  M  C  T  R  V  T  S      40

AAAATCATCCCTTCCACCGAGGATCCTAATGAGGACATTGTGGAGAGAAATATCCGAATT   180
K  I  I  P  S  T  E  D  P  N  E  D  I  V  E  R  N  I  R  I      60

GTTGTCCCTTTGAACAACAGGGAGAATATCTCTGATCCCACCTCCCCACTGAGAAGGAAC   240
V  V  P  L  N  N  R  E  N  I  S  D  P  T  S  P  L  R  R  N      80

TTTGTATACCATTTGTCAGACGTCTGTAAGAAATGCGATCCTGTGGAAGTGGAGCTGGAA   300
F  V  Y  H  L  S  D  V  C  K  K  C  D  P  V  E  V  E  L  E     100

GATCAGGTTGTTACTGCCACCCAGAGCAACATCTGCAATGAAGACGATGGTGTTCCTGAG   360
D  Q  V  V  T  A  T  Q  S  N  I  C  N  E  D  D  G  V  P  E     120

ACCTGCTACATGTATGACAGAAACAAGTGCTATACCACTATGGTCCCACTTAGGTATCAT   420
T  C  Y  M  Y  D  R  N  K  C  Y  T  T  M  V  P  L  R  Y  H     140

GGTGAGACCAAAATGGTGCAAGCAGCCTTGACCCCCGATTCTTGCTACCCTGACTGACTC   480
G  E  T  K  M  V  Q  A  A  L  T  P  D  S  C  Y  P  D          160

GAGATT
```

Figure 1a: The nucleotide and deduced amino acid sequence of the murine J chain PCR product. The underlined nucleotides indicate location of restriction sites.

5 BRADFORD PROTEIN ASSAY

Protein concentrations were determined by using a dye-binding assay (Bradford) according to manufacturer's instructions. 25 µl of sample was added to 1 ml of Coomassie plus™ protein assay reagent (Pierce, UK) and the Absorbance 595 nm was then measured. Absorbency values were compared to a standard curve determined for Bovine Serum Albumen.

6 ABBREVIATIONS

ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BIAcore	BIAcore biosensor ™
BIP	Binding protein
β–ME	β–mercaptoethanol
BSA	Bovine serum albumen
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
CDR	Complementarity-determining region
CHO	Chinese hamster ovary
CPMV	Cowpea mosaic virus
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
GPCR	G-protein coupled receptor
GRP7	Glucose regulating protein
HA	Haemagglutinin
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HSP	Heat shock protein
HSV	Herpes simplex virus
LPS	Lipopolysaccharide-specific
MAB	Monoclonal antibody
MDCK	Madin-Darby canine kidney
MHC	Major histocompatability complex
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PDI	Protein disulphide isomerase
pIgR	Poly-immunoglobulin receptor
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SA I/II	Streptococcal antigen I/II
SC	Secretory component
scFv	Single-chain Fv antibody fragment
SDS -PAGE	Sodium dodecyl sulphate/ polyacrylamide gel electrophoresis
SIgA	Secretory IgA
STD	Sexually transmitted disease
T-DNA	Transfer DNA
TMV	Tobacco mosaic virus
TSP	Total soluble protein
UDP	Uridine 5' diphosphate
UPR	Unfolded protein response
UTP	Uridine 5' triphosphate
UV	Ultraviolet
VSV	Vesicular stomatitis virus
WT	Wild type

PUBLISHED PAPERS

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ER–resident chaperone interactions with recombinant antibodies in transgenic plants

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In this study, we demonstrate that the folding and assembly of IgG in transgenic tobacco plants is orchestrated by BiP (binding protein), an endoplasmic reticulum resident chaperone. Expression of BiP and calreticulin was examined in transgenic tobacco plants that express immunoglobulin chains, either singly or in combination to form IgG antibody. BiP mRNA expression was lowest in wild-type nontransformed plants and those that expressed immunoglobulin light chain alone. Higher mRNA levels were detected in plants expressing fully assembled immunoglobulin (light and heavy chains), and the most abundant levels of RNA transcript were found in those plants that expressed immunoglobulin heavy chain alone. Estimation of total BiP demonstrated a similar pattern, with the highest levels detected in plants expressing immunoglobulin heavy chain alone. Immunoprecipitation studies demonstrated that BiP was associated with immunoglobulin chains extracted from protoplast lysates, but not from secreted fluids. Again, most BiP was coprecipitated from plants expressing heavy chain only and those that produced full length IgG. The binding of BiP to Ig heavy chains was ATP-sensitive. Co-expression of heavy and light chain resulted in IgG assembly and displacement of BiP from the heavy chain as the amount of light chain increased. Although calreticulin mRNA and total protein levels varied in a similar manner to those of BiP in the transgenic plants, there was no evidence for association between calreticulin and Ig chains, by coimmunoprecipitation. The results indicate that BiP, but not calreticulin, takes part in immunoglobulin folding and assembly in transgenic plants.

Keywords: BiP; IgG; transgenic plants; immunoglobulin assembly; chaperones.

A wide variety of functional recombinant antibody molecules have been expressed successfully in transgenic plants, ranging from small monomeric fragments [1–3] to full length IgG [2,4,5] as well as more complex multimeric secretory antibodies [6]. The synthesis, folding and assembly of complex mammalian proteins, such as full length immunoglobulins (Igs) in plants can be extremely efficient, resulting in expression levels of between 1 and 5% of total plant protein [4,6,7], that compare favourably with mammalian hybridoma cell culture. Protein folding and assembly within cells is a complex process with stringent quality control mechanisms (reviewed in [8]). It is largely regulated by enzymes and an array of molecular chaperones. In mammalian and plant cells, the best characterized chaperone is BiP (binding protein), a lumenal endoplasmic reticulum (ER) resident member of the heat shock protein 70 family of stress proteins [9]. BiP has been identified in various mammals [10–13], yeast [14,15] and plants [16,17]. By binding to newly synthesized polypeptides, BiP is thought to stabilize partially folded intermediates during folding and assist in the assembly of protein oligomers [18]. BiP also has other functions in protein translocation into the ER, prevention and dissolution of protein aggregates and retention of misfolded or unassembled subunit proteins [18,19]. Plant BiP shares approximately 69% homology with mammalian BiP at the amino acid level [16] and is similarly involved in assisting the folding of plant proteins [17,20]. BiP is also found in association with assembly defective proteins in plants [21,22].

Calreticulin is a highly conserved protein also found in the ER and nuclear envelope [23]. It is the major calcium binding protein in the ER [24] and also appears to act as a storage site for BiP [25]. Calreticulin is a stress-induced protein [26] and shares several regions of sequence homology (42–78%) with the chaperone calnexin [27,28]. Its own role as a chaperone has been demonstrated in the folding and assembly of major histocompatibility (MHC) class I molecules [29] and the envelope glycoprotein from human immunodeficiency virus [30]. As with calnexin, calreticulin binds specifically to glycosylated proteins, and in at least one example (HIV gp160) both calnexin and calreticulin are associated with the newly synthesized molecule [30].

In mammalian cells, the interactions between immunoglobulin chains and chaperones have been partially

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Abbreviations: BiP, binding protein; CR, calreticulin; MHC, major histocompatibility complex; UPR, unfolded protein response; WT, wild-type.

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characterized. Newly synthesized heavy and light chains associate with BiP immediately after synthesis [31,32]. The interaction is brief but BiP displays a strong preference for early folding intermediates over mature molecules. Thereafter, a relay of chaperones is likely to be involved [33], including GRP94 and possibly GRP170 [34], as well as protein disulphide isomerase [35].

Relatively little is known about ER chaperones in plants. Limited evidence is available for the interaction of BiP and CR (calreticulin) with newly synthesized endogenous and defective polypeptides, whereas a systematic study of chaperone interaction with heterologous proteins has not been performed. The aims of this study were to determine how the plant ER responds to the expression of heterologous secretory proteins. We therefore studied the involvement of BiP and calreticulin in the folding and assembly of recombinant immunoglobulin heavy and light chains in transgenic plants that express the assembled form of an IgG monoclonal antibody or individual IgG heavy or light chains. If those chaperones were to be actively involved in antibody processing, this might explain the efficiency and relatively high yield of functional antibody production that can be achieved in plants.

EXPERIMENTAL PROCEDURES

Transgenic plants

Three homozygous, transgenic, *Nicotiana tabacum* plant lines that have been described previously were used in this study [5]. These plant lines expressed the light chain of a murine IgG1 antibody alone (κ), the heavy chain alone (γ), or both κ and γ chains (IgG). Nontransformed wild-type (WT) plants were used as a control. The transgenes were introduced into the tobacco plants using agrobacterium [36], under the control of the CaMV 35S promoter and a mouse immunoglobulin leader sequence to target the gene products for secretion through the ER. Transgenic plants coexpressing immunoglobulin light and heavy chains were generated by cross-fertilization between parent plants. The plants used in this study were grown under sterile conditions.

Western blotting and ELISA

To confirm the expression of each immunoglobulin chain, leaf extracts were examined by Western blot analysis, using specific antisera [5]. For total BiP assay, 8 mm leaf punches were taken from 6-week-old-plants. Samples were extracted in NaCl/Tris, pH 8, with 10 mg·mL^{-1} leupeptin (Calbiochem). Aliquots of the protein extracts were separated by SDS/PAGE under reducing conditions and blotted onto nitrocellulose membranes. The membranes were blocked in NaCl/Tris containing 0.05% (v/v) Tween 20 and 1% (w/v) nonfat dry milk, and then incubated with a rabbit anti-(tobacco BiP) antiserum (kindly supplied by J. Denecke, Leeds University, Leeds, UK) for 2 h at 37 °C. Bound antibody was detected using an alkaline phosphatase–conjugated goat anti-(rabbit IgG) serum (Sigma, UK) in conjunction with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad, UK) detection reagents.

Capture ELISAs were used to quantify recombinant protein expression in transgenic plants. ELISA plates (Nunc Immobilon, UK) were coated with either anti-(mouse κ) (Caltag, USA), or anti-(mouse IgG1) (gamma 1 chain specific) (The Binding Site, UK) at a concentration of 5 μg·mL^{-1}. For IgG detection, the plates were incubated with purified streptococcal antigen at a concentration of 2 μg·mL^{-1}. Plates were incubated for 16 h at 4 °C, washed twice in sterile distilled water and blocked by addition of 2.5% (w/v) BSA in NaCl/P_i with a 2 h incubation at 37 °C. For the assay, leaf extracts were centrifuged (20 000 *g*, 10 min, 4 °C) and aliquots added in duplicate to the ELISA plate wells. Plates were incubated for 16 h at 4 °C after which they were washed five times with distilled water with 0.5% (v/v) Tween 20. Secondary antibody was an affinity-purified HRPO-conjugated anti-(mouse κ) (Caltag, USA) or γ serum (The Binding Site, UK) as appropriate. Detection was with tetramethylbenzidine dihydrochloride peroxidase substrate (Sigma, UK). Colour development was allowed to proceed for 10 min, then 25 μL of 2 M H_2SO_4 were added to each well and the absorbance read at 450 nm on an ELISA plate reader (Anthos, UK).

RNA extraction

Total RNA was extracted from young plants two months after germination, essentially as described by Logemann *et al.* [37]. Briefly, leaf tissue was frozen in liquid nitrogen and rapidly ground to a fine powder. This was mixed with two vols of ice cold guanidine hydrochloride buffer (8 M guanidine hydrochloride, 20 mM Mes, 20 mM EDTA, 50 mM 2-mercaptoethanol, pH 7). After agitation it was added to one vol of phenol : chloroform : isoamyl alcohol (25 : 24 : 1, v/v/v), mixed thoroughly, and centrifuged at 12 000 *g* for 45 min. The aqueous phase was collected, mixed with ethanol (0.7 volumes) and 1 M acetic acid (0.2 vols), and incubated at −20 °C for 16 h. After centrifugation, the precipitate was washed three times with 3 M sodium acetate, pH 5.2, and once with 70% ethanol. The pellet was dissolved in sterile RNAse free water (Sigma, UK) containing RNAse free DNAse (Promega, UK), and incubated at 37 °C for 1 h, then at 70 °C for 5 min. RNA concentration and purity were assessed using the GeneQuant II RNA/DNA Calculator (Pharmacia, UK).

DNA probe preparation

The plasmid pGEM3Z (Promega) containing a 2420 bp DNA fragment (BLP4) from the tobacco homologue of BiP was kindly provided by J. Denecke. A 1400 bp DNA fragment from the castor bean calreticulin gene cloned into Bluescript KS$^+$ (Stratagene, USA) was kindly supplied by S. Coughlan, Du Pont Agrochemicals, USA [38]. *Escherichia coli* DH5α (GibcoBRL) was transformed and plasmid DNA was prepared using a commercially available kit (Qiagen). Plasmids were linearized and probe DNA was labeled with [α-^{32}P]dCTP using a commercially available kit (dCTP, Ready To Go™, Pharmacia Biotech). Labeled DNA was denatured by boiling for 3 min in 1 vol formamide before use.

Northern blotting

Fifteen μg of total plant leaf RNA were prepared in 10× Mops buffer, 5% (v/v) formaldehyde, and 50% (v/v) formamide. Samples were heated at 60 °C for 10 min, and

run on a 1.2% (w/v) agarose gel prepared with 2.2 M formaldehyde. The gel was stained in ethidium bromide, then soaked for 30 min in 50 mM $NaOH/NaH_2PO_4$, 5 mM EDTA, rinsed in RNAse-free water and soaked for 45 min in 20× $NaCl/P_i$/EDTA (3.6 M NaCl, 0.2 M Na_2HPO_4, 20 mM EDTA, pH 6.5). The RNA was then transferred to a Hybond N^+ membrane (Amersham) using a transfer pyramid with 20× $NaCl/P_i$/EDTA as the buffer. Following 16 h transfer, the membrane was air-dried for 45 min and the RNA fixed by irradiation with a UV Crosslinker (Hoefer Scientific Instruments). Confirmation of uniform RNA transfer was by visualization of ethidium bromide staining under UV illumination. In addition, a positive control nontransgenic flower pooled RNA sample was included on the left and right sides of each gel.

For probing, the membrane was incubated in prehybridization buffer [50% (v/v) formamide, 5× $NaCl/P_i$/EDTA, 1% (w/v) SDS, 0.1% (w/v) sodium tetraborate, 50 $mg \cdot L^{-1}$ heparin] at 42 °C overnight. Denatured probe was added for 16 h at 42 °C. The final probe concentration was 5 $ng \cdot mL^{-1} \cdot kb^{-1}$ of probe complexity equaling 10^6–10^7 $dpm \cdot ml^{-1}$. The membrane was washed with 0.1× $NaCl/P_i$/EDTA, 0.5% (w/v) SDS at room temperature, and then twice at 42 °C, for 30 min with gentle shaking. The membrane was washed once with 0.1× $NaCl/P_i$/EDTA at room temperature and blotted dry, prior to exposure on Biomax MR film (Kodak Scientific Imaging) at −70 °C, for 72 h.

Protoplasts isolation and transfection

Protoplasts were prepared from the leaves of 4- to 6-week-old-tobacco as described by Otsuki *et al.* [39] Protoplasts were subjected to polyethylene glycol-mediated transfection exactly as described by Pedrazzini *et al.* [22] and incubated overnight at 25 °C in the dark before pulse labeling. Pulse-chase labeling of protoplasts using Pro-Mix (a mixture of [^{35}S]Met and [^{35}S]Cys; Amersham) was performed as described [21]. Cell fractionation and microsome preparation were also performed as described [7]. Homogenization of protoplasts was performed by adding to the frozen samples two vols of ice-cold homogenization buffer [150 mM Tris/HCl, 150 mM NaCl, 1.5 mM EDTA and 1.5% (v/v) Triton X-100, pH 7.5] supplemented with Complete (Boehringer) protease inhibitor cocktail.

Immunoprecipitation

Immunoprecipitation of expressed polypeptides from labeled protoplasts was performed as described previously [21], using rabbit polyclonal antisera raised against mouse IgG (Sigma) or BiP [22]. Immunoselected proteins were analyzed by 15% reducing SDS/PAGE and fluorography.

For unlabelled plant protoplasts, cell homogenates from 2×10^6 cells from each plant line were incubated with a goat anti-(mouse IgG) serum. Immunoselected polypeptides were analyzed under both reducing and nonreducing conditions on SDS/PAGE and blotted onto nitrocellulose membranes. The membranes were used either in autoradiograph or immunoblot detection as described above, using a rabbit anti-(plant BiP) serum or a goat anti-(mouse IgG) serum and appropriate second-layer alkaline phosphatase-conjugated antibodies.

RESULTS

Confirmation of transgenic gene product expression in plant lines

Stable homozygous seed stocks were used to generate the plants used in this study. Six plants representing each line were selected and the expression of transgenic gene product (none, κ chain, γ chain or both chains) was examined by appropriate ELISA and Western blot. All transgene products were of the expected relative molecular mass (not shown), as previously reported [5]. Within each group of plants, there appeared to be no significant difference in expression levels, as measured by the intensity of immunoreactive bands on Western blot (data not shown). A capture ELISA confirmed four plants in each group that had identical titration curves, with the other two plants differing by one or two dilution steps (Fig. 1). Subsequent experiments were performed using plants that expressed equivalent levels of recombinant protein within each group, to eliminate the effect of different levels of expression of each construct. The relative expression levels between groups were calculated from ELISA results, as a percentage of total soluble protein by comparison with known Ig standards and these were (mean ± SD): κ, 0.007% ± 0.001; γ, 0.207% ± 0.023 and IgG, 1.27% ± 0.21.

Detection of BiP mRNA in transgenic plants

Northern blot analysis of transgenic plants using a BiP DNA probe is shown in Fig. 2. Flower and leaf tissue from six transgenic plants representing each construct were used and results from two plants in each group are shown. The positive control nontransgenic flower pooled RNA sample indicated uniform transfer of RNA onto the blot in both cases (not shown). A transcript of the expected size was found in all plant lines in both flowers (Fig. 2A) and leaves (Fig. 2B). Overall, the BiP transcript was more prominent in flower samples as expected [16]. Between lines, the levels of hybridizing transcripts varied, with a consistent pattern between flower and leaf samples. BiP transcript was higher in those plants expressing the heavy chain alone, and hardly detectable (at the levels of film exposure shown here) in the wild-type plants or those expressing light chain alone. An intermediate level of transcript expression was found in plants expressing assembled IgG. Densitometry of the bands using the positive control nontransgenic flower pooled RNA sample as a standard demonstrated a significant difference between wild-type and Gamma plants, and wild-type and IgG plants, but not between gamma and IgG plants (not shown).

The results are in agreement with previous findings that BiP is constitutively expressed in flowers, and at lower levels in mature leaf tissue [16], but suggest that BiP expression is increased when the plant cells are actively synthesizing secretory proteins, such as recombinant Ig heavy chain or assembled immunoglobulin. However, BiP mRNA levels do not correlate with overall recombinant protein expression levels, as although IgG plants express approximately sixfold more recombinant protein than the respective heavy chain plants, the BiP mRNA level is lower. A possible explanation is that BiP only operates on unassembled chains and is

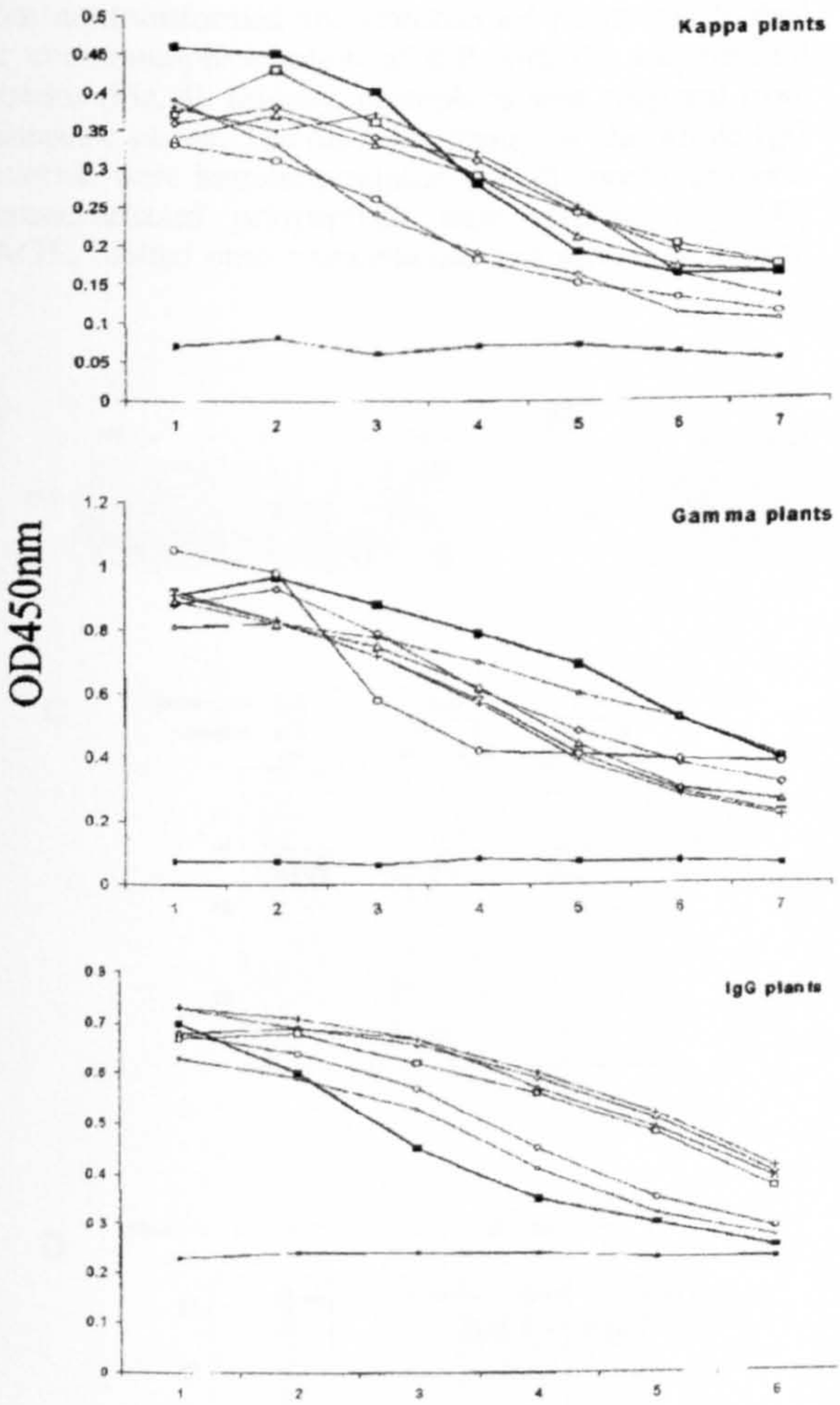

Fig. 1. Titration curves for transgenic plant extracts. Six transgenic plants expressing either antibody light (κ) chain, heavy (γ) chain or both chains were assayed by capture ELISA. For the light and heavy chain ELISAs, capture was with the relevant specific goat antiserum and detection was with a horseradish peroxidase labeled goat anti-kappa or gamma serum. For functional IgG assay, capture was with the specific antigen (streptococcal antigen I/II at 2 $\mu g \cdot mL^{-1}$) and detection was with a horseradish peroxidase labeled goat anti-gamma serum. Controls were Guy's 13 IgG hybridoma cell culture supernatant and an extract from a wild-type nontransformed plant. The titration curves for all samples are shown (mean of duplicate wells). Positive and negative control samples are shown as large and small black squares, respectively. Samples from the two plants that differed from the other four and were not used in further studies (see main text) are shown as large and small open circles.

gradually displaced upon heavy-light chain assembly (see further below).

Total BiP protein expression in transgenic plants

Western blotting was used to compare total BiP protein in crude extracts prepared from wild-type plants and two plants each expressing unassembled light chains, unassembled heavy chains or assembled immunoglobulin. The

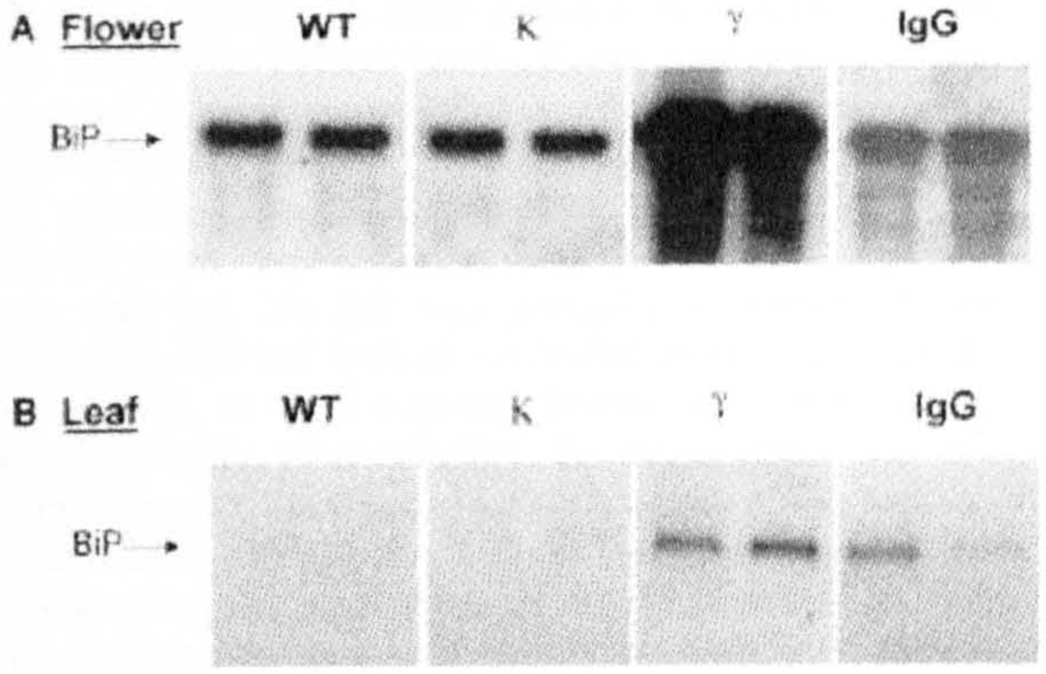

Fig. 2. Northern blot analysis of plant RNA. Fifteen μg total RNA from leaf tissue was run in each lane, blotted onto nitrocellulose and probed with a labeled anti-BiP DNA probe. The expected position of BiP transcript is indicated. Each of the four panels in each set is taken from the same nitrocellulose blot and probed in an identical manner. WT, wild type nontransformed tobacco; κ, kappa chain transgenic tobacco; γ, gamma chain transgenic tobacco; and IgG, kappa and gamma chain transgenic tobacco.

protein immunoblot is shown in Fig. 3. As a control, the crude extract from the flower of a wild type plant was used to demonstrate the expected position of BiP on Western blot (Fig. 3, lane 1). The flower extract was used as a marker solely because BiP protein levels are normally higher in flowers [16]. As expected, a band of approximate M_r of 75 000 was detected, and no cross-reactive bands were found. Using the same mass of starting leaf material, the lowest level of total BiP was detected in leaf extracts from wild-type plants, and similar levels were observed in plants that expressed unassembled κ chain. The highest levels of BiP protein were detected in leaf extracts from plants expressing unassembled γ heavy chains, and those that expressed assembled IgG.

The immunoblotting was repeated using all six transgenic plants in each group with the same results (not shown).

Co-immunoprecipitation of BiP with immunoglobulin chains in plants

To investigate the specific association of BiP with recombinant immunoglobulin chains, we prepared protoplasts

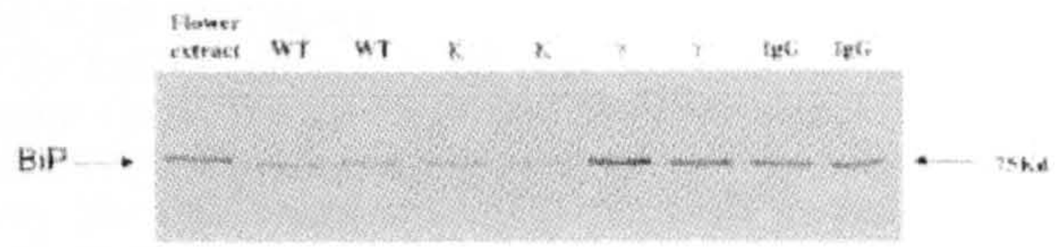

Fig. 3. Western blot analysis of total BiP protein in plant extracts. Leaf extracts were separated by SDS/PAGE and blotted onto nitrocellulose. The filter was probed with anti-BiP serum, followed by an alkaline phosphatase conjugated anti-rabbit IgG serum. A flower extract was used as a positive control. Results from two plants representing each plant line are shown. WT, wild type nontransformed tobacco; κ, kappa chain transgenic tobacco; γ, gamma chain transgenic tobacco; and IgG, kappa and gamma chain transgenic tobacco.

from nontransformed and transformed plants and looked for coimmunoprecipitation of BiP with the recombinant proteins (Fig. 4). Initially, protoplasts were prepared from transgenic plants. The antibody chains or the whole IgG molecule were immunoprecipitated with specific antisera. Immunoselected polypeptides were resolved by SDS/PAGE, blotted onto nitrocellulose and probed with anti-BiP serum. Using the same number of protoplasts from each plant, the highest level of coprecipitating BiP was recovered from transgenic plants expressing only heavy chains (Fig. 4A, γ). Less BiP was coprecipitated from plants that produced assembled immunoglobulin (IgG), and a faint BiP band was detected from plants expressing light chain only (κ). No BiP was precipitated from wild type plants that did not express recombinant immunoglobulin chains (WT), or from a sample consisting of immunoprecipitation buffer alone (buffer). To demonstrate that BiP interaction with immunoglobulin chains was an intracellular phenomenon and not due to nonspecific interactions, the immunoprecipitation experiment was also carried out using protoplast lysates in comparison with protoplast culture medium (Fig. 4B). The anti-BiP serum did not cross-react with Guy's 13 IgG. BiP was detected in immunoprecipitates from IgG transgenic protoplasts, but not from the protoplast medium, even though IgG, which is normally secreted [40], was detected by ELISA in this sample (not shown).

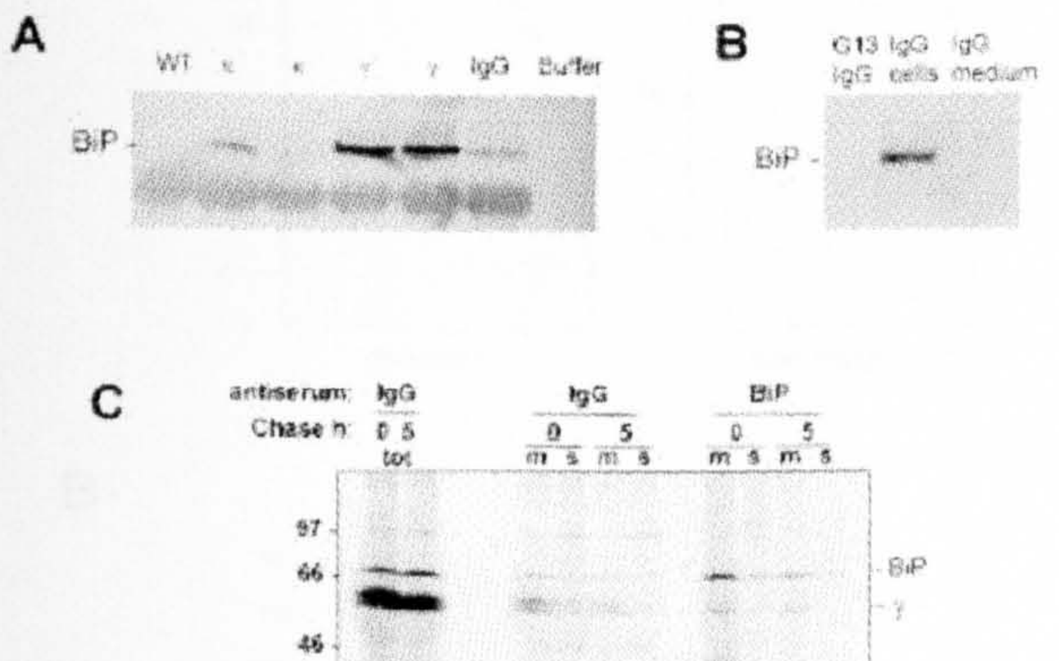

Fig. 4. Western blot analysis of immunoprecipitates from plant protoplasts. The source material was transgenic plants (A,B) or transiently transformed protoplasts (C). A and B: the blots were probed with rabbit anti-BiP serum followed by alkaline phosphatase conjugated anti-rabbit serum. WT, wild type nontransformed tobacco; κ, kappa chain transgenic tobacco; γ, gamma chain transgenic tobacco; IgG, kappa and gamma chain transgenic tobacco; buffer, NET buffer containing the goat antiserum used for immunoprecipitation; G13 IgG, Guy's 13 IgG hybridoma cell culture supernatant; IgG cells, protoplast extract from IgG plants; and IgG medium, culture medium from IgG protoplasts. (C) protoplasts were transfected with plasmid encoding the IgG heavy chain, pulse labelled for 1 h and chased for 5 h. Total cell homogenates (tot) or microsomal (m) and soluble (s) fractions were immunoprecipitated with anti-IgG or anti-BiP. Numbers at left indicate molecular mass markers in kDa: protoplasts were transfected with plasmids encoding the light chain (k), the heavy chain (γ) or both chains (IgG), pulse labeled for 1 h and homogenized. Cell homogenates were immunoprecipitated with the indicated antisera. Bands are visualized by autoradiography. The four panels represent cells expressing IgG, γ, κ or control. Immunoprecipitation was with anti-κ, anti-IgG or anti-BiP antisera as indicated. Immunoprecipitates were incubated with (+) or without (–) 4 mM ATP prior to SDS/PAGE.

To assess whether coprecipitation of IgG heavy chain with BiP was a result of the proteins colocalizing and not a posthomogenization artefact, we subjected transgenic protoplasts to metabolic labelling for 1 h, homogenized them in 12% (w/v) sucrose and purified microsomes [7]. Figure 4C shows that both IgG and BiP are retrieved in the microsomal fraction by immunoprecipitation with either anti-IgG or anti-BiP sera. The interaction between BiP and the heavy chain is prolonged, as both polypeptides are still coselectable after 5 h chase (Fig. 4C).

To confirm that coprecipitation of BiP reflected real chaperone action, we tested whether the interaction of BiP with immunoglobulin chains was sensitive to ATP. For these experiments, protoplasts that transiently expressed the recombinant proteins were used. The cells were pulse-labeled for 1 h and cell homogenates were immunoselected with either anti-BiP or antisera specific for single IgG chains. The results confirmed the different levels of coimmunoprecipitating BiP in γ- and IgG- expressing cells (Fig. 4D). However, in these assays there was no evidence of BiP coimmunoprecipitating with κ chain. The results also demonstrate that BiP was released from immunoselected gamma chain by washing with 4 mM ATP, suggesting a ligand–chaperone relationship between the two molecules. Note that immunoprecipitation with anti BiP antiserum in all panels of Fig. 4D leads to coselection of an unrelated polypeptide of the same size of the γ chain. Coselection of this polypeptide is insensitive to ATP treatment. The presence of this contaminant band partly explains why ATP release of γ chain from BiP does not seem complete in the 'γ' and 'IgG' panels.

To further prove that BiP interacts with unassembled heavy chains, we reasoned that the coexpression of the companion κ chain should compete with BiP for association with the IgG heavy chain. We therefore cotransfected tobacco protoplasts with a fixed amount of DNA encoding heavy chain and with increasing amounts of light-chain encoding DNA, then immunoprecipitated the immunoglobulin chain (Fig. 5). The results show that when heavy chain (γ) expression is constant, an increase in light chain (κ) expression is paralleled by a decrease in the amount of BiP that is coselected from the cell homogenates. When the same samples were run on nonreducing SDS/PAGE, it was clear that cotransfection of the light chain resulted in the

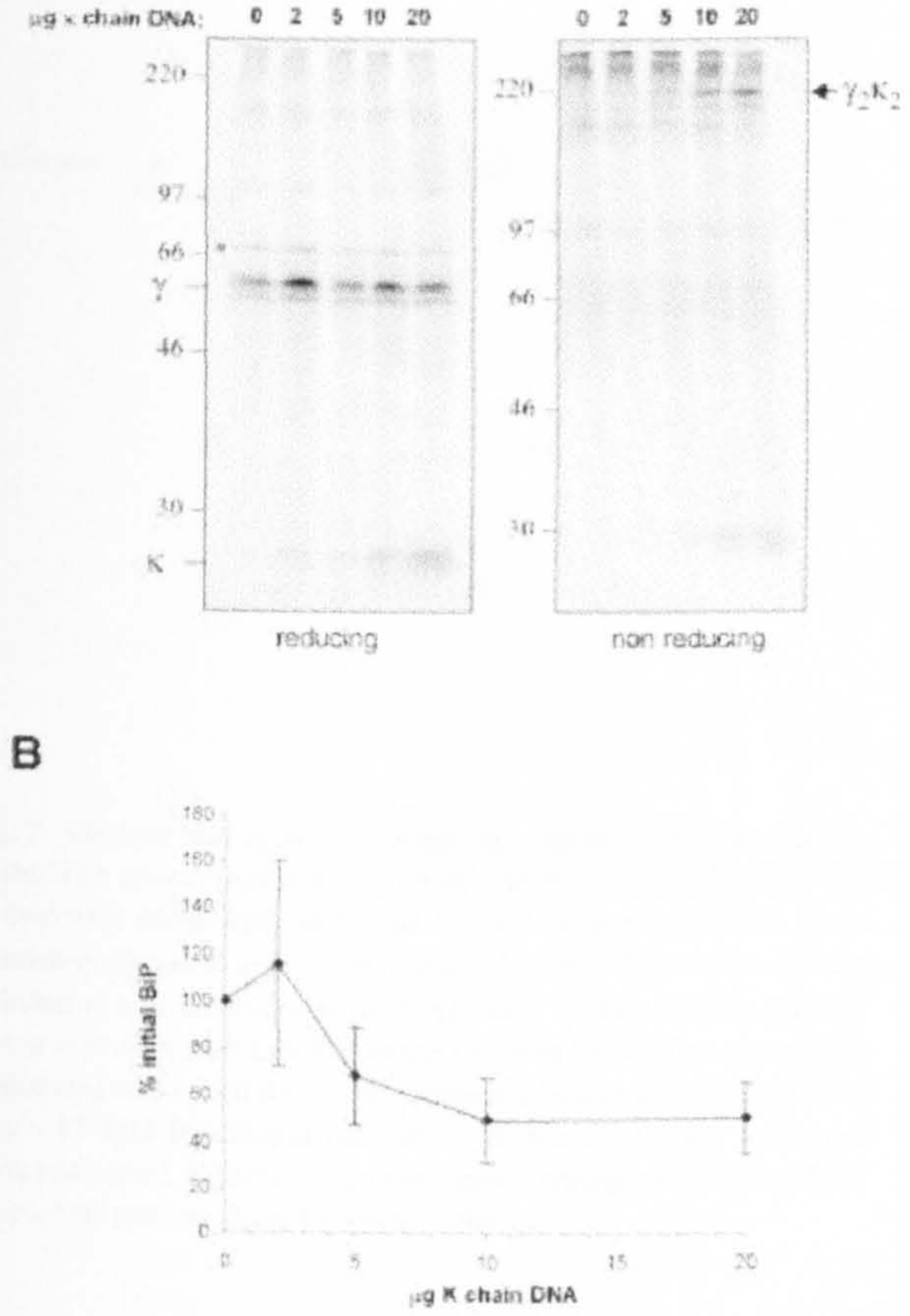

Fig. 5. Co-expression of κ chain competes with BiP for binding to the heavy chain. (A) Protoplasts were cotransfected with a constant amount (40 μg) of plasmid encoding heavy chain and with the indicated amounts of light chain. Cells were pulse labeled for 1 h and homogenized. Cell homogenates were immunoselected with anti IgG or anti BiP antisera, resolved by reducing or nonreducing SDS/PAGE and polypeptides visualized by fluorography. The panel shows the result from one of four independent experiments. Numbers at left indicate molecular mass markers in kDa. (B) The intensity of the immunoselected BiP bands in A was evaluated by densitometry. The results shown are the average of four independent experiments. Bars indicate standard deviation.

assembly of the IgG heterotetramer in a dose-dependent manner (Fig. 5A). Therefore, the presence of the light chain triggers assembly of the IgG tetramers and causes BiP to be partially released from the heavy chains.

Detection of calreticulin mRNA and protein in transgenic plants

RNA extracted from the leaves of six plants representative of each construct was used in Northern hybridizations with a calreticulin specific DNA probe, and the results from two plants are shown (Fig. 6). The results mirrored those of BiP (Fig. 2), with the levels of hybridizing transcripts being highest in plants expressing the heavy chain alone, and lowest in the wild type plants or those expressing light chain

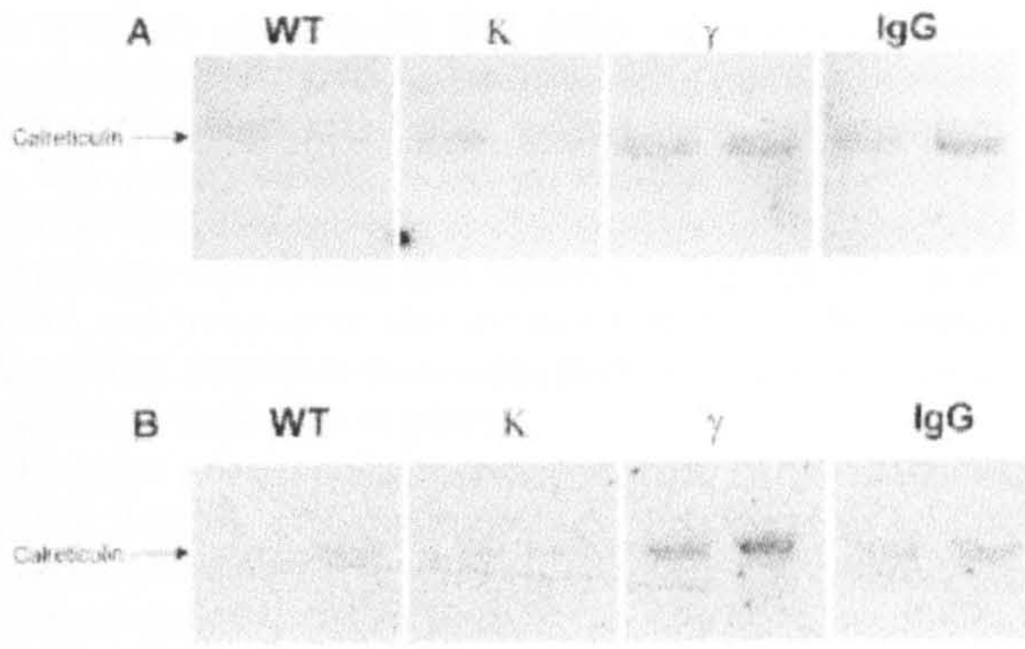

Fig. 6. Northern blot analysis of plant RNA. Fifteen μg total RNA from leaf tissue was run in each lane, blotted onto nitrocellulose and probed with a labeled anticalreticulin DNA probe. The expected position of calreticulin transcript is indicated. Each of the four panels in each set is taken from the same nitrocellulose blot and probed in an identical manner. WT, wild type nontransformed tobacco; κ, kappa chain transgenic tobacco; γ, gamma chain transgenic tobacco; and IgG, kappa and gamma chain transgenic tobacco.

alone, with an intermediate level of transcript expression found in plants expressing assembled IgG.

Western blot analysis of total calreticulin protein expression levels in the different plants also demonstrated a pattern similar to that found for BiP. The lowest levels were detected in wild-type extracts and plants expressing κ chain alone. Higher levels of calreticulin were detected in plants expressing assembled IgG and the highest expression levels were in plants expressing unassembled immunoglobulin heavy chains (not shown).

Co-immunoprecipitation of calreticulin with plant-expressed immunoglobulin chains

In contrast to BiP, it was not possible to detect calreticulin in association with immunoglobulin chains (Fig. 7). Protoplasts were prepared from nontransformed (WT) and transformed (γ and IgG) plants. Lysed cell extracts were immunoprecipitated with antiserum to murine IgG (heavy and light chains), and after SDS/PAGE and immunoblotting, detection was with either anti-calreticulin (A) or anti-BiP (B) antisera. No coprecipitating bands of the expected size for calreticulin were detected from any plant (Fig. 7A), as compared with calreticulin present in a WT flower extract. However, as shown previously, coprecipitating BiP was detected from the same heavy chain γ plant sample but not WT (Fig. 7B).

DISCUSSION

A number of expression systems have been used to produce antibody molecules. For full-length antibodies, bacterial systems are inappropriate, due to the demands of protein glycosylation and assembly, but IgG can be expressed in yeast [41] and in baculovirus/insect cell systems [42,43]. In mammalian cells, the importance of ER resident chaperones in the assembly of immunoglobulins has been recognized for some time [9]. The best characterized of these is BiP (binding protein or GRP78 [44]), which was initially shown to bind Ig

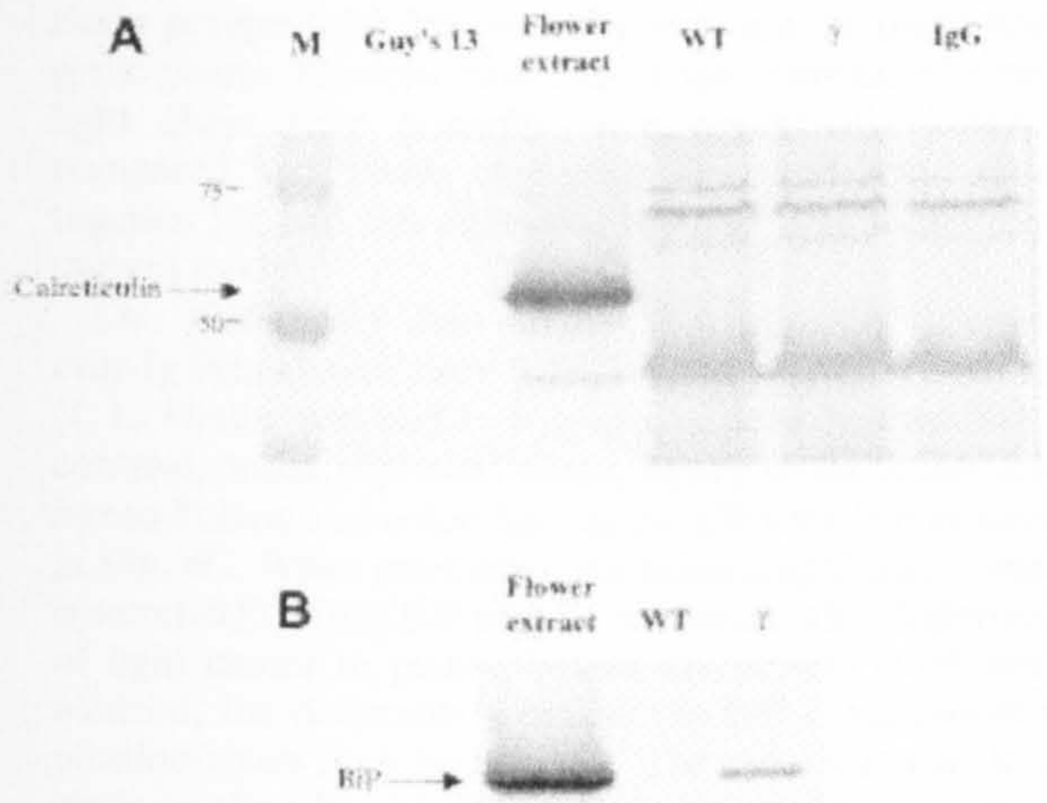

Fig. 7. Western blot analysis of immunoprecipitates from plant protoplasts. The source material was transgenic plants. (A) The blot was probed with rabbit anti-calreticulin serum followed by alkaline phosphatase–conjugated anti-rabbit serum. A crude flower extract was included as a positive control for recognition by the antibody. (B) The blot was probed with anti-BiP serum followed by alkaline phosphates conjugated anti-rabbit serum. M, protein molecular size markers; G13, Guy's 13 IgG hybridoma cell culture supernatant; WT, wild type nontransformed tobacco; γ, gamma chain transgenic tobacco; IgG, kappa and gamma chain transgenic tobacco.

heavy chains, and later light chains (see [33] for review). Association with Ig chains occurs immediately after synthesis, and usually lasts for a matter of minutes if there are no abnormal folding or assembly events [31,34]. A number of BiP binding sites have been mapped within the antibody [45] and dissociation of BiP is ATP-dependent [34]. The successful assembly of IgG in yeast and insect cell systems may be attributable to the presence of appropriate protein chaperones, as yeast possesses a homologue of BiP, termed Kar2 [15,46]. Although a native BiP has not been identified in insect cell lines, candidate insect protein chaperones have been identified that may perform a similar role [47]. Indeed, when insect cells were engineered to express murine BiP, increased expression of functional antibodies was found alongside a decrease in the formation of abnormal protein aggregates [48].

One of the potential advantages of the plant expression system is the presence of an indigenous BiP which is highly conserved as compared with murine BiP, with approximately 69% overall homology at the amino acid level. This compares with yeast BiP which has 64% overall homology with murine BiP. In tobacco, BiP mRNA expression is highest in tissues containing rapidly dividing cells or those that are involved in secretion [16], whereas in maize, BiP is expressed most abundantly in endosperm development [17]. Also, plant BiP expression is associated with the accumulation of abnormal proteins [21]. It was previously demonstrated that efficient assembly and expression of Igs in plants could only be achieved by using a leader sequence to target the recombinant immunoglobulin proteins to the ER and the secretory pathway [4]. This might be due to enhanced translation of recombinant proteins or to increased stability of the proteins resulting from their subcellular localization. In this paper, several lines of evidence have been put forward that demonstrate the association of the plant BiP homologue with folding and assembly of Ig light and heavy chains and we propose that the involvement of ER-resident chaperones promotes processing and expression of immunoglobulin molecules in plants.

Previous analysis of the constitutive expression patterns of BiP mRNA has suggested that expression is low in tobacco leaves [16]. For this reason, in these investigations we have used leaf tissues initially, so that any elevation in BiP would be more readily detected over the low background of constitutive expression. BiP mRNA was differentially expressed in four plant lines that expressed no transgenic product, Ig light or heavy chain, or assembled IgG. The highest BiP expression was found in plants expressing heavy chain only, BiP expression was relatively lower in plants that express both light and heavy chains, but still elevated as compared with nontransgenic plants. The results are consistent with the putative role of BiP in binding to and retaining unassembled subunit proteins [9,49] and in folding and assembly of immunoglobulin chains in cells that are highly active in terms of recombinant protein production and secretion. The relative decrease in BiP mRNA in IgG expressing plants as compared with heavy chain expressing plants might be attributable to the successful assembly of heavy chains into immunoglobulin. This reduces the levels of nonassembled heavy chain, and allows the release and recycling of BiP. This is reflected in the total extractable BiP from leaf tissue, which was elevated to similar levels in both γ and IgG transgenic plants.

BiP protein coprecipitated with immunoglobulin chains extracted from protoplast intracellular fluid, but not from secreted Ig chains. This indicates that the BiP–Ig chain interaction in plants is not artefactual and is consistent with the model of BiP binding transiently in the ER to Ig chains during protein folding and processing. The immunoblot results matched those found by Northern blot, in that most BiP protein was immunoprecipitated in plants expressing the Ig heavy chain only. Less was detected from plants expressing both light and heavy chains, even though more Ig heavy chain protein is consistently recoverable from IgG-expressing plants. Again this is consistent with the model for BiP in assisting protein folding and increasing throughput of the ER. In mammals, BiP associates with both Ig light and heavy chains [9,31]. The evidence for BiP interaction with heavy chains in plants is clear, however, inconclusive results were obtained for the involvement of BiP with light chain. When analysis was performed using samples derived from transgenic plants expressing light chain only, a faintly discernible BiP band was immunoprecipitated. However, no such interaction was observed when light chain was transiently expressed in protoplasts. The difference in results may be due to a difference in expression levels of immunoglobulin light chain between the two systems. Alternatively, it may reflect a more rapid turnover of transiently expressed light chains, which would lead to its interaction with the pool of unlabelled BiP within the time frame of our observation. In view of the finding that BiP mRNA expression was not elevated in single transgenic plants, an alternative explanation is that the steady-state levels of light

chain polypeptides are generally very low in single transgenic plants. Previous estimates of the expression levels of light chain have generally been significantly lower as compared with plants expressing light and heavy chains together [4], and this difference has also been found in the current study.

Our preliminary data suggest that in plants expressing only Ig light chains, these light chains are normally secreted (J. L. Hadlington and L. Frigerio, unpublished results). In contrast, when expressed alone, heavy chain accumulates intracellularly and colocalizes in the ER with BiP as shown in Fig. 4C. When assembled, the immunoglobulin complex is secreted [7]. Thus BiP may be involved in the chaperoning of light chains in plants, but as the protein is efficiently secreted, the detectable signal in the BiP coimmunoprecipitation assay may be too low. The association of heavy chain in the ER with BiP is likely to represent chaperone activity, as indicated by the fact that upon addition of further recombinant protein (i.e. light chain), the BiP levels decreased, rather than increased. We cannot formally exclude that the BiP levels observed result from an unfolded protein response (UPR), but a detailed analysis of UPR markers in our transgenic plants is beyond the scope of this work.

In plants, BiP has been shown to interact transiently with the monomeric form of the storage protein phaseolin. Upon trimerization of phaseolin, BiP is released, indicating that it plays a role in trimer assembly [20]. Only when a mutant of phaseolin is incapable of forming trimers is it found in prolonged association with BiP, until it is eventually degraded by quality control [21,22]. Similarly, we show here that BiP is tightly associated to single heavy chains, but the association is less strong when both heavy and light chains are present simultaneously. Moreover, BiP association is reduced when increasing amounts of light chain are available for IgG assembly.

The association of BiP with folding and assembly of recombinant immunoglobulin chains in plants is significant in demonstrating the suitability and potential versatility of transgenic plants for producing a variety of mammalian proteins. Protein translocation and folding in the ER can be one of the rate limiting steps in protein secretion, and the presence of protein chaperones is important for high efficiency turnover, leading to high levels of production. It has been recently reported that the overexpression of BiP (and PDI) in yeast cells greatly improves the efficiency of folding and secretion of single chain antibody fragments [50]. Likewise, when BiP is overexpressed in transgenic plants, it is able to alleviate ER stress induced by tunicamycin [51]. It will be very interesting to test the effects of BiP overexpression in plants expressing our model Igs.

The passage of proteins through the secretory pathway in plants is a complicated process [52] and it is clear that BiP is not the only chaperone involved. In mammals, a few other chaperones that are involved with Igs have been identified so far [33,34]. Of these, plant homologues to GRP 94, and protein disulphide isomerase [53] have been identified, and it will be important to establish their role in Ig assembly. In this study, we have investigated whether calreticulin might be involved in the folding and assembly of immunoglobulins in plants and found no evidence for this. Although we cannot exclude the possibility that an extremely rapid interaction occurs between calreticulin and immunoglobulin chains, our findings appear to be consistent with the mammalian expression of immunoglobulins [33], and demonstrates a specific and appropriate interaction between Igs and chaperones in plants. With increasing evidence for separate chaperone pathways involving either BiP or calreticulin/calnexin [54] our demonstration that the BiP pathway in plants is functional for mammalian proteins provides a functional rationale for the use of plants as an expression system. It remains to be determined if the plant calreticulin pathway is equally functional for mammalian proteins. Conversely, the range of transgenic plants now available that express all combinations of Ig chains involved in IgG and SIgA may be useful tools to investigate immunoglobulin processing and help to identify candidate proteins which may act as chaperones in this process.

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REFERENCES

1. Benvenuto, E., Ordas, R.J., Tavazza, R., Ancora, G., Biocca, S., Cattaneo, A. & Galeffi, P. (1991) 'Phytoantibodies': a general vector for the expression of immunoglobulin domains in transgenic plants. *Plant Mol Biol.* **17**, 865–874.
2. De Neve, M., De Loose, M., Jacobs, A., Van Houdt, H., Kaluza, B., Weidle, U. & Depicker, A. (1993) Assembly of an antibody and its derived antibody fragment in Nicotiana and Arabidopsis. *Transgenic Res.* **2**, 227–237.
3. Owen, M., Gandecha, A., Cockburn, B. & Whitelam, G. (1992) Synthesis of a functional anti-phytochrome single-chain Fv protein in transgenic tobacco. *Biotechnology (NY)* **10**, 790–794.
4. Hiatt, A., Cafferkey, R. & Bowdish, K. (1989) Production of antibodies in transgenic plants. *Nature* **342**, 76–78.
5. Ma, J.K.-C., Lehner, T., Stabila, P., Fux, C.I. & Hiatt, A. (1994) Assembly of monoclonal antibodies with IgG1 and IgA heavy chain domains in transgenic tobacco plants. *Eur. J. Immunol.* **24**, 131–138.
6. Ma, J.K.-C., Hiatt, A., Hein, M., Vine, N.D., Wang, F., Stabila, P., von Dolleweerd, C., Mostov, K. & Lehner, T. (1995) Generation and assembly of secretory antibodies in plants. *Science* **268**, 716–719.
7. Frigerio, L., Vine, N.D., Pedrazzini, E., Hein, M.B., Wang, F., Ma, J.K.-C. & Vitale, A. (2000) Assembly, secretion and vacuolar delivery of a hybrid immunoglobulin in plants. *Plant Physiol.* **123**, 1483–1493.
8. Ellgaard, L., Molinari, M. & Helenius, A. (1999) Setting the standards: quality control in the secretory pathway. *Science* **286**, 1882–1888.
9. Haas, I.G. & Wabl, M. (1983) Immunoglobulin heavy chain binding protein. *Nature* **306**, 387–389.
10. Haas, I.G. & Meo, T. (1988) cDNA cloning of the immunoglobulin heavy chain binding protein. *Proc. Natl Acad. Sci. USA* **85**, 2250–2254.
11. Munro, S. & Pelham, H.R.B. (1986) An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell* **46**, 291–300.
12. Ting, J., Wooden, S.K., Kriz, R., Kelleher, K., Kaufman, R.J. & Lee, A.S. (1987) The nucleotide sequence encoding the hamster 78-kDa glucose-regulated protein (GRP78) and its conservation between hamster and rat. *Gene* **55**, 147–152.

13. Wooden, S.K., Kapur, R.P. & Lee, A.S. (1988) The organization of the rat GRP78 gene and A23187-induced expression of fusion gene products targeted intracellularly. *Exp Cell Res.* **178**, 84–92.
14. Normington, K., Kohno, K., Kozutsumi, Y., Gething, M.-J. & Sambrook, J. (1989) S. cerevisiae encodes an essential protein homologous in sequence and function to mammalian BiP. *Cell* **57**, 1223–1236.
15. Rose, M.D., Misra, L.M. & Vogel, J.P. (1989) *KAR2*, a karyogamy gene, is the yeast homolog of the mammalian BiP/GRP78 gene. *Cell* **57**, 1211–1221.
16. Denecke, J., Goldman, M.H.S., Demolder, J., Seurinck, J. & Botterman, J. (1991) The tobacco luminal binding protein is encoded by a multigene family. *Plant Cell* **3**, 1025–1035.
17. Fontes, E.B.P., Shank, B.B., Wrobel, R.L., Moose, S.P., Obrian, G.R., Wurtzel, E.T. & Boston, R.S. (1991) Characterization of an immunoglobulin binding protein homolog in the maize floury-2 endosperm mutant. *Plant Cell* **3**, 483–496.
18. Gething, M.-J. (1999) Role and regulation of the ER chaperone BiP. *Sem. Cell Dev. Biol.* **10**, 465–472.
19. Nishikawa, S., Fewell, S.W., Kato, Y., Brodsky, J.L. & Endo, T. (2001) Molecular chaperones in the yeast endoplasmic reticulum maintain the solubility of proteins for retrotranslocation and degradation. *J. Cell Biol.* **153**, 1061–1069.
20. Vitale, A., Bielli, A. & Ceriotti, A. (1995) The binding protein associates with monomeric phaseolin. *Plant Physiol.* **107**, 1411–1418.
21. Pedrazzini, E., Giovinazzo, G., Bollini, R., Ceriotti, A. & Vitale, A. (1994) Binding of BiP to an assembly-defective protein in plant cells. *Plant J.* **5**, 103–110.
22. Pedrazzini, E., Giovinazzo, G., Bielli, A., de Virgilio, M., Frigerio, L., Pesca, M., Faoro, F., Bollini, R., Ceriotti, A. & Vitale, A. (1997) Protein quality control along the route to the plant vacuole. *Plant Cell* **9**, 1869–1880.
23. Denecke, J., Carlsson, L.E., Vidal, S., Höglund, A.-S., Ek, B., van Zeijl, M.J., Sinjorgo, K.M.C. & Palva, E.T. (1995) The tobacco homolog of mammalian calreticulin is present in protein complexes in vivo. *Plant Cell* **7**, 391–406.
24. Michalak, M., Milner, R.E., Burns, K. & Opas, M. (1992) Calreticulin. *Biochem. J.* **285**, 681–692.
25. Crofts, A.J., Leborgne-Castel, N., Pesca, M., Vitale, A. & Denecke, J. (1998) BiP and calreticulin form an abundant complex that is independent of endoplasmic reticulum stress. *Plant Cell* **10**, 813–823.
26. Park, B.J. & Lee, D.G., YuJ.R., Jung, S.K., Choi, K., Lee, J., Kim, Y.S., Lee, J.I., Kwon, J.Y., Singson, A., Song, W.K., Eom, S.H., Park, C.S., Kim, D.H., Bandyopadhyay, J. & Ahnn, J. (2001) Calreticulin, a calcium-binding molecular chaperone, is required for stress response and fertility in Caenorhabditis elegans. *Mol Biol. Cell* **12**, 2835–2845.
27. Crofts, A.J. & Denecke, J. (1998) Calreticulin and calnexin in plants. *Trends Plant Sci.* **3**, 396–399.
28. Hochstenbach, F., David, V., Watkins, S. & Brenner, M.B. (1992) Endoplasmic reticulum resident protein of 90 kilodaltons associates with the T- and B-cell antigen receptors and major histocompatibility complex antigens during their assembly. *Proc. Natl Acad. Sci. USA* **89**, 4734–4738.
29. Sadasivan, B., Lehner, P.J., Ortmann, B., Spies, T. & Cresswell, P. (1996) Roles for calreticulin and a novel glycoprotein, tapasin, in the interaction of MHC class I molecules with TAP. *Immunity* **5**, 103–114.
30. Otteken, A. & Moss, B. (1996) Calreticulin interacts with newly synthesized human immunodeficiency virus type 1 envelope glycoprotein, suggesting a chaperone function similar to that of calnexin. *J. Biol. Chem* **271**, 97–103.
31. Knittler, M.R. & Haas, I.G. (1992) Interaction of BiP with newly synthesized immunoglobulin light chain molecules: cycles of sequential binding and release. *EMBO J.* **11**, 1573–1581.
32. Vanhove, M., Usherwood, Y.K. & Hendershot, L.M. (2001) Unassembled Ig heavy chains do not cycle from BiP *in vivo* but require light chains to trigger their release. *Immunity* **15**, 105–114.
33. Melnick, J. & Argon, Y. (1995) Molecular chaperones and the biosynthesis of antigen receptors. *Immunol. Today* **16**, 243–250.
34. Melnick, J., Dul, J.L. & Argon, Y. (1994) Sequential interaction of the chaperones BiP and GRP94 with immunoglobulin chains in the endoplasmic reticulum. *Nature* **370**, 373–375.
35. Roth, R.A. & Pierce, S.B. (1987) *In vivo* cross-linking of protein disulfide isomerase to immunoglobulins. *Biochemistry* **26**, 4179–4182.
36. Fraley, R.T., Rogers, S.G., Horsch, R.B., Sanders, P.R., Flick, J.S., Adams, S.P., Bittner, M.L., Brand, L.A., Fink, C.L., Fry, J.S., Galluppi, G.R., Goldberg, S.B., Hoffmann, N.L. & Woo, S.C. (1983) Expression of bacterial genes in plant cells. *Proc. Natl Acad. Sci. USA* **80**, 4803–4807.
37. Logemann, J., Schell, J. & Willmitzer, L. (1987) Improved method for the isolation of RNA from plant tissues. *Anal Biochem.* **163**, 16–20.
38. Coughlan, S.J., Hastings, C. & Winfrey, R.J. (1997) Cloning and characterization of the calreticulin gene from *Ricinus communis* L. *Plant Mol Biol.* **34**, 897–911.
39. Otsuki, Y., Takebe, I., Honda, Y. & Matsui, C. (1972) Ultrastructure of infection of tobacco mesophyll protoplasts by tobacco mosaic virus. *Virology* **49**, 188–194.
40. Frigerio, L. & Lord, J.M. (2000) Glycoprotein degradation: do sugars hold the key? *Curr. Biol.* **10**, R674–R677.
41. Horwitz, A.H., Chang, C.P., Better, M., Hellstrom, K.E. & Robinson, R.R. (1988) Secretion of functional antibody and Fab fragment from yeast cells. *Proc. Natl Acad. Sci. USA* **85**, 8678–8682.
42. Hasemann, C.A. & Capra, J.D. (1990) High-level production of a functional immunoglobulin heterodimer in a baculovirus expression system. *Proc. Natl Acad. Sci. USA* **87**, 3942–3946.
43. zu Putlitz, J., Kubasek, W.L., Duchene, M., Marget, M., von Specht, B.U. & Domdey, H. (1990) Antibody production in baculovirus-infected insect cells. *Biotechnology (NY)* **8**, 651–654.
44. Haas, I.G. (1991) BiP – a heat shock protein involved in immunoglobulin chain assembly. *Curr. Top Microbiol. Immunol.* **167**, 71–82.
45. Knarr, G., Gething, M.J., Modrow, S. & Buchner, J. (1995) BiP binding sequences in antibodies. *J. Biol. Chem* **270**, 27589–27594.
46. Vogel, J.P., Misra, L.M. & Rose, M.D. (1990) Loss of BiP/GRP78 function blocks translocation of secretory proteins in yeast. *J. Cell Biol.* **110**, 1885–1895.
47. Jarvis, D.L., Oker-Blom, C. & Summers, M.D. (1990) Role of glycosylation in the transport of recombinant glycoproteins through the secretory pathway of lepidopteran insect cells. *J. Cell Biochem.* **42**, 181–191.
48. Hsu, T.A., Eiden, J.J., Bourgarel, P., Meo, T. & Betenbaugh, M.J. (1994) Effects of co-expressing chaperone BiP on functional antibody production in the baculovirus system. *Protein Expr Purif* **5**, 595–603.
49. Knittler, M.R., Dirks, S. & Haas, I.G. (1996) Molecular chaperones involved in protein degradation in the endoplasmic reticulum: quantitative interaction of the heat shock cognate protein BiP with partially folded immunoglobulin light chains that are degraded in the endoplasmic reticulum. *Proc. Natl Acad. Sci. USA* **92**, 1764–1768.
50. Shusta, E.V., Raines, R.T., Plückthun, A. & Wittrup, K.D. (1998) Increasing the secretory capacity of Saccharomyces cerevisiae for production of single-chain antibody fragments. *Nat Biotechnol.* **16**, 773–777.
51. Leborgne-Castel, N., Jelitto-Van Dooren, E.P.W.M., Crofts, A.J. & Denecke, J. (1999) Overexpression of BiP in tobacco alleviates endoplasmic reticulum stress. *Plant Cell* **11**, 459–469.

52. Vitale, A. & Galili, G. (2001) The endomembrane system and the problem of protein sorting. *Plant Physiol.* **125**, 115–118.
53. Shorrosh, B.S. & Dixon, R.A. (1991) Molecular cloning of a putative plant endomembrane protein resembling vertebrate protein disulfide-isomerase and a phosphatidylinositol-specific phospholipase C. *Proc. Natl Acad. Sci. USA* **88**, 10941–10945.
54. Molinari, M. & Helenius, A. (2000) Chaperone selection during glycoprotein translocation into the endoplasmic reticulum. *Science* **288**, 331–333.

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Assembly and plasma membrane targeting of recombinant immunoglobulin chains in plants with a murine immunoglobulin transmembrane sequence

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Abstract

The cDNA encoding a full-length murine immunoglobulin $\gamma 1$ heavy chain with its native leader sequence, transmembrane and intracellular domains was introduced into transgenic plants. Transformed plants expressed the recombinant polypeptide, but, in contrast to plants expressing the heavy chain without transmembrane sequence, the protein appeared to be associated with a plant cell membrane. Extraction of the membrane-associated heavy chain required the presence of a non-ionic detergent, and immunofluorescence studies of protoplasts demonstrated surface expression of membrane Ig heavy chain on up to 40% of the cells from a transgenic leaf. In plants expressing both the membrane Ig heavy chain and its partner light chain, functional antibody was also localised to the plant cell membrane and retention of the heavy chain at this site appeared to have no effect on the efficiency of antibody assembly. This approach of localising and accumulating recombinant antibody in cell membranes may have a number of applications, including passive immunisation against plant pathogens.

Introduction

The expression of full-length antibodies and antibody fragments in transgenic plants is an important tool in plant science which can be used for a number of purposes (Ma and Hiatt, 1996). A particular advantage of antibodies is the diversity of engineered molecular derivatives that can be generated and retain antigen binding and specificity. Thus expression of a single-chain molecule (scFv) has been used to modify plant biochemistry (Owen *et al.*, 1992) and to induce resistance to disease (Tavladoraki *et al.*, 1993). They can be expressed in the cytoplasm, targeted into the secretory pathway and retained in the endoplasmic reticulum (ER) or secreted (Firek *et al.*, 1993; Artsaenko *et al.*, 1995; Schouten *et al.*, 1996). However, there are disadvantages, as scFv molecules bind antigen monovalently and therefore cannot cause aggregation; moreover, they bind less avidly than their parent antibodies. The expression levels of scFv are often disappointing, up to 0.1% total soluble protein when expressed in the cytosol, although this can be increased by targeting the scFv to the ER (Firek *et al.*, 1993) or by retention in the endoplasmic reticulum using the KDEL sequence (Schouten *et al.*, 1996). In contrast, full-length antibodies which bind antigen bivalently are generally expressed at high levels in transgenic plants (Hiatt *et al.*, 1989; Ma *et al.*, 1994). For the correct assembly of functional full-length immunoglobulins, the individual heavy and light chains need to be targeted to the endoplasmic reticulum and plant secretory pathway (Hiatt *et al.*, 1989).

Although the final cellular distribution of recombinant antibodies has not been fully characterised, a significant amount is accumulated in the apoplastic space, which is a relatively stable environment (Hein *et al.*, 1991). The subsequent fate of these antibodies is also

unknown and they may be degraded, be transported elsewhere in the plant, or even be reinternalised.

In mammalian B lymphocytes and plasma cells, immunoglobulin molecules exist in two forms: as secreted antibody or as a surface antigen receptor. The membrane-associated forms have heavy chains that are slightly larger than those of secreted antibody due to the presence of an extra 71 amino acid residues at the COOH terminus. These are arranged in 3 domains, a 17 residue acidic extracellular portion, a 26 residue hydrophobic intra-membrane portion and a 28 residue hydrophilic intracellular portion (Yamakawi-Kataoka *et al.*, 1982). These sequences are highly conserved within the murine Cγ1, Cγ2a and Cγ2b as well as Cμ genes. The aims of this study were (1) to determine if a murine immunoglobulin heavy chain could be targeted to the plant cell membrane using the native transmembrane sequence, (2) to examine if correctly assembled functional antibody could be retained at this site, and (3) to determine the effect of this sequence on the expression of Ig heavy chain and assembled IgG in transgenic tobacco plants.

Materials and methods

DNA cloning and sequencing

In order to clone the DNA encoding the membrane-associated immunoglobulin heavy chain (mγ1), messenger RNA was first purified from the Guy's 13 hybridoma cell line by acid-guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). Guy's 13 is a murine IgG1 monoclonal antibody that was raised against a streptococcal surface antigen (I/II) (Smith and Lehner, 1989). cDNA was prepared with MMLV reverse transcriptase (Promega, UK). DNA encoding the heavy chain, with its native leader and transmembrane sequences, was amplified by polymerase chain reaction (PCR) with synthetic oligonucleotides: GGGCTCGAGATGRRATGSASCTGGRTYKTNYTC (5′) and TGTGAATTCCTAGGGCGCTTGCCCAAT (3′). The membrane-associated heavy-chain gene was ligated into pBluescript (Stratagene, USA) for sequence analysis by the dideoxy chain termination procedure (Sequenase, USB, USA). The gene was transferred to a constitutive plant expression vector (pMON 530) which contains the 35S CaMV promoter sequence (Rogers *et al.*, 1987). The recombinant vector was used to transform *Escherichia coli* strain DH5-α (Gibco-BRL, UK). Screening for positive transformants was done by Southern blotting with a radiolabelled DNA probe derived from the original PCR product. Plasmid DNA was purified and introduced into *Agrobacterium tumefaciens* as described (Fraley et al. 1983).

Transformation and regeneration of transgenic plants

Leaf discs of *Nicotiana tabacum* (var. *xanthii*) were transformed as described (Horsch *et al.*, 1985). The discs were incubated on medium supplemented with kanamycin (200 mg/l) and carbenicillin (500 mg/l) for regeneration of shoots, and the shoots were transferred to root-inducing medium supplemented with kanamycin (200 mg/l). As soon as roots were established, plantlets were transplanted into soil.

Transgenic plants expressing the secreted form of the Guy's 13 heavy chain and its partner light chain have already been prepared and described (Ma *et al.*, 1994). Plants expressing assembled immunoglobulin were prepared by cross-fertilisation between heavy-chain- (secreted or membrane-associated forms) and light-chain-expressing plants as described previously, and screened by an antigen-specific ELISA.

Immunoglobulin chain detection

Young plant leaves were homogenised in 150 mM NaCl, 20 mM Tris-HCl pH 8 (TBS) containing leupeptin (10 μg/ml, Calbiochem, USA). 1% Nonidet P40 (NP40) was included as indicated. After centrifugation at 13 000 × *g* for 2 min, the supernatant was added in 3-fold dilutions to microtitre plates that had previously been coated either with goat anti-mouse γ1 antiserum (1:1000, Sigma, UK) or streptococcal antigen I/II (2 μg/ml) and blocked with 5% non-fat dry milk in TBS containing 0.05% Tween 20 (TBST). Incubation was done for 2 h at 37 °C, the plates were then washed three times with TBST buffer. Bound immunoglobulin chains were detected by incubation with horseradish peroxidase-labelled goat anti-mouse γ1 antiserum (1:1000, Nordic Pharmaceuticals, Netherlands) for 2 h at 37 °C, followed by ABTS (2.2′-azino-di-(3-ethyl-benzthiazoline sulfonate), Boehringer Mannheim, FRG) as the substrate. Antibody concentration was calculated by comparison of binding curves with pre-existing standards. Soluble protein concentration of total plant extracts was measured with the BCA protein assay reagent (Pierce, USA).

Preparation of protoplasts

Sterile tobacco leaf sections were incubated overnight in 0.5 M mannitol, 50 mM $CaCl_2$, 3 mM MES (mannitol buffer) containing 0.4% cellulase and 0.2% pectinase (Calbiochem, UK) pH 5.4 at room temperature in the dark. Gross debris was removed and the protoplast suspension centrifuged at 65 × *g* for 10 min. The floating layer of cells was removed by pipette, washed three times in four volumes of buffer containing 150 mM NaCl, 5 mM KCl, 125 mM $CaCl_2$ and 5 mM glucose (W5 buffer), then resuspended at ca. 2 × 10^6 protoplasts/ml. Protoplasts were not fixed, but maintained essentially as live cells.

Immunofluorescent labelling of protoplasts

Either FITC-labelled goat anti-mouse κ chain (1:25, Sigma-Aldrich, UK) or FITC-labelled goat anti-mouse g1 antiserum (1:25, Sigma-Aldrich, UK) were added to protoplasts and incubated for 2 h at room temperature in the dark. Protoplasts were then washed 3 times by centrifugation at 65 × *g* in W5 buffer and viewed by fluorescence microscopy.

For control samples, protoplasts from a plant transformed with an irrelevant protein were incubated for 2 h at room temperature with a polyclonal rabbit antiserum against *Arabidopsis* plasma membrane proton pump ATPase (1:100), kindly provided by Dr Mike Sussman (University of Wisconsin). Protoplasts were washed 3 times in W5 buffer prior to incubation with FITC goat anti-rabbit IgG (1:25, Sigma-Aldrich, UK) for a further 2 h, and then washed a further 3 times in W5 buffer.

ELISA analysis of protoplasts

Protoplast supernatants from overnight digestion were collected and applied to microtitre plates as described. The protoplasts were also harvested on a sucrose cushion, resuspended in mannitol buffer and disrupted by sonication. The samples were centrifuged at 50 000 rpm for 45 min at 4 °C. Cell pellets were resuspended in TBS, leupeptin and 1% NP40 and applied to microtitre plates as described previously.

Guy's 13 MAb Membrane Spanning Region

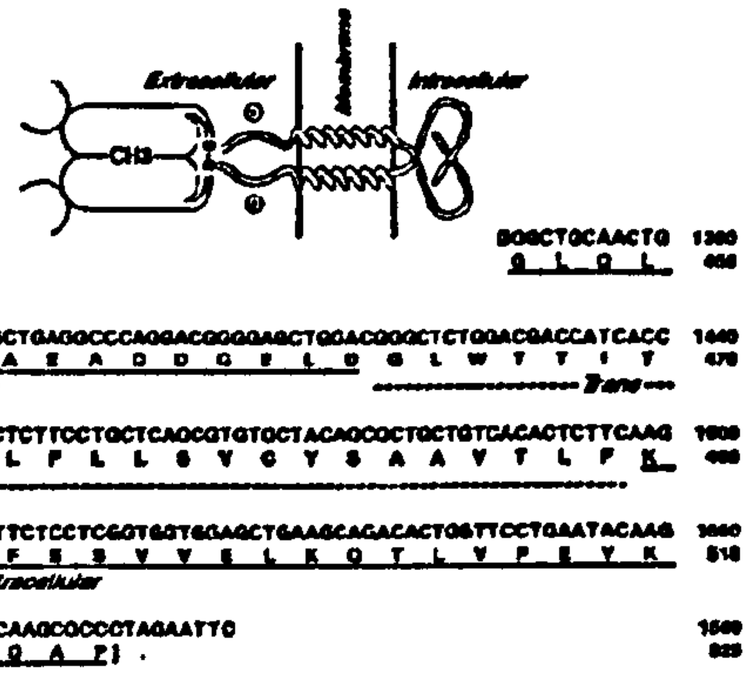

Figure 1. Diagrammatic representation of the C-terminus of my1 and predicted amino acid sequence of Guy's 13 my1 heavy chain. The functional regions are indicated.

Results

Cloning, sequencing of the immunoglobulin heavy chain and generation of transgenic plants

DNA encoding the entire heavy chain of a murine IgG1 monoclonal antibody including its native leader and transmembrane sequences was amplified by PCR using cDNA prepared from the hybridoma cell line. A degenerate 5′ oligonucleotide primer and a specific 3′ primer were used that were designed from previously published sequences (Yamawaki-Kataoka *et al.*, 1982; Kabat *et al.*, 1999). A PCR product of the expected size (ca. 1589 bp) was amplified and cloned. Sequence anaylsis identified the region encoding the native signal sequence MEWTWVFLFLLSGTAGVIIS and confirmed the DNA sequence of the extracellular domains of the Guy's 13 heavy chain. The sequence of the 3′ end of the DNA fragment is shown in Figure 1 and is identical to that previously published for the 2PK-3 membrane IgG1 (Tyler *et al.*, 1982).

The cloned product was introduced into transgenic tobacco plants by *Agrobacterium*-mediated transformation. A number of plants were regenerated, from which twelve independent transgenic plants were identified with an Ig heavy chain-specific ELISA (data not shown). Four were used for cross-fertilisation with transgenic plants already expressing the Guy's 13 light chain (Ma *et al.*, 1994). Of 86 progeny plants screened by an antigen-specific ELISA described below, 28 expressed functional Guy's 13 antibody.

Detection of recombinant Ig chains by ELISA

Representative data from plants expressing Guy's 13 membrane-associated heavy chain (mγ1) with or without Guy's 13 light chain are shown (Figure 2). Addition of NP40 had no effect on the extraction of Guy's 13 heavy chain from plants not expressing the transmembrane region (IgG) (Figure 2A). However, in those plants expressing mγ1, the heavy chain could only be detected if NP40 was present in the extraction buffer. In plants expressing mγ1 with Guy's 13 light (κ) chain (mIgG) some heavy chain was detected in the absence of NP40, but the titre was 2–3 dilution steps less than when NP40 was present.

In these latter plants expressing both mγ1 and κ chains, the antibody was correctly assembled in the plant cell membrane, as demonstrated by an antigen-specific ELISA (Figure 2B). As before, antibody was detected at a level 2–3 dilution steps higher in the presence of NP40. The titration curves for IgG activity and heavy-chain expression were similar in these plants, indicating that there was little free heavy-chain present and that most was assembled into functional antibody. No antigen binding was observed in extracts from plants expressing mγ1 alone.

As indicated in Figure 2B, the titration curves for total heavy-chain detection in mIgG plants are about the same as those for mγ1 plants (Figure 2A). Indeed, there was only a 2.5-fold difference in mean titres measured from 12 plants each expressing mγ1 or mIgG (results not shown). The mean recovery for mγ1 and mIgG was 0.5% and 1.1% of total soluble plant protein respectively.

Protoplast analysis

Detection of mγ1 heavy chain from protoplast preparations by ELISA is shown in Figure 2C. The recombinant heavy chain was detectable in mγ1 plants in the ultracentrifuged membrane pellet resuspended in buffer containing NP40, but not the ultracentrifuged supernatant from the cell wall digestion buffer suggesting that recombinant mγ1 is not secreted. Similar results were found for mIgG plants, although there were low levels of functional antibody detected in the supernatant. As before, the levels of mγ1 detection were similar between mγ1 and mIgG plants. There was little or no detectable immunoglobulin heavy chain in the membrane preparations from transgenic plants expressing the soluble form of IgG only, although there were detectable levels of secreted antibody in the culture supernatant.

Immunofluorescence of protoplasts to demonstrate membrane-associated heavy chain is shown in Figures 3 and 4. The majority of protoplasts appeared intact, although some were slightly misshapen, probably due to the fact that the protoplasts were prepared without fixation. It is also possible that cross-linking of membrane proteins by the specific antibodies might affect the morphology of the cells. Protoplasts were labelled with an FITC anti-mouse γ-chain antiserum. Protoplasts are autofluorescent (red) under UV illumination due to chlorophyll (Figure 3A, panels 1 and 2), and this fluorescence is limited to chloroplasts. Cells from untransformed (WT) plants were not stained with FITC (panel 3). About 1 in 30 protoplasts from untransformed plants exhibited weak fluorescence but there were no moderately or brightly stained cells. In contrast, cells from the mγ1 plants demonstrate bright surface staining (Figure 3B, panel 1) which is distinct from the chloroplasts (Figure 3B, panels 2 and 3). The numbers of fluorescing cells in the mγ1 leaf preparations varied between 10% and 40%. In the most highly fluorescing preparations, ca. 20% of the cells were brightly fluorescent, a further 20% had moderate fluorescence and the remainder emitted minimal or no fluorescent signals. The fluorescence pattern associated with protoplasts expressing mγ1 was similar to that seen when protoplasts were incubated with an antiserum to detect plasma membrane H(+)-ATPase, i.e. a native plasma membrane marker (Figure 3C, panels 3 and 4). In this case, protoplasts were prepared from a control transformed plant expressing an irrelevant secreted antigen. A further control was included to exclude the possibility of non-specific binding by the FITC-labelled goat anti-rabbit Ig antiserum (Figure 3C, panels 1 and 2).

Immunofluorescence of protoplasts to demonstrate membrane-associated antibody is shown in Figure 4. Protoplasts were labelled with either FITC anti-mouse κ-chain or FITC anti-mouse γ-chain antisera. There was no specific staining of cells from untransformed plants (WT) or those only expressing the mγ1 chain (Figure 4A, panels 1–4) with the anti-κ reagent. The mγ1 cells, however, were positively stained (as before) for anti-γ-chain (Figure 4B, panels 3 and 4). This demonstrates the specificity of the anti-κ-chain reagent, and absence of cross-reactivity with immunoglobulin heavy chain. Protoplasts from plants co-expressing mγ1 and κ did demonstrate surface staining both with anti-κ (Figure 4A, panels 5 and 6) and anti-γ (Figure 4B, panels 1 and 2) antisera. This confirms the presence of surface immunoglob-

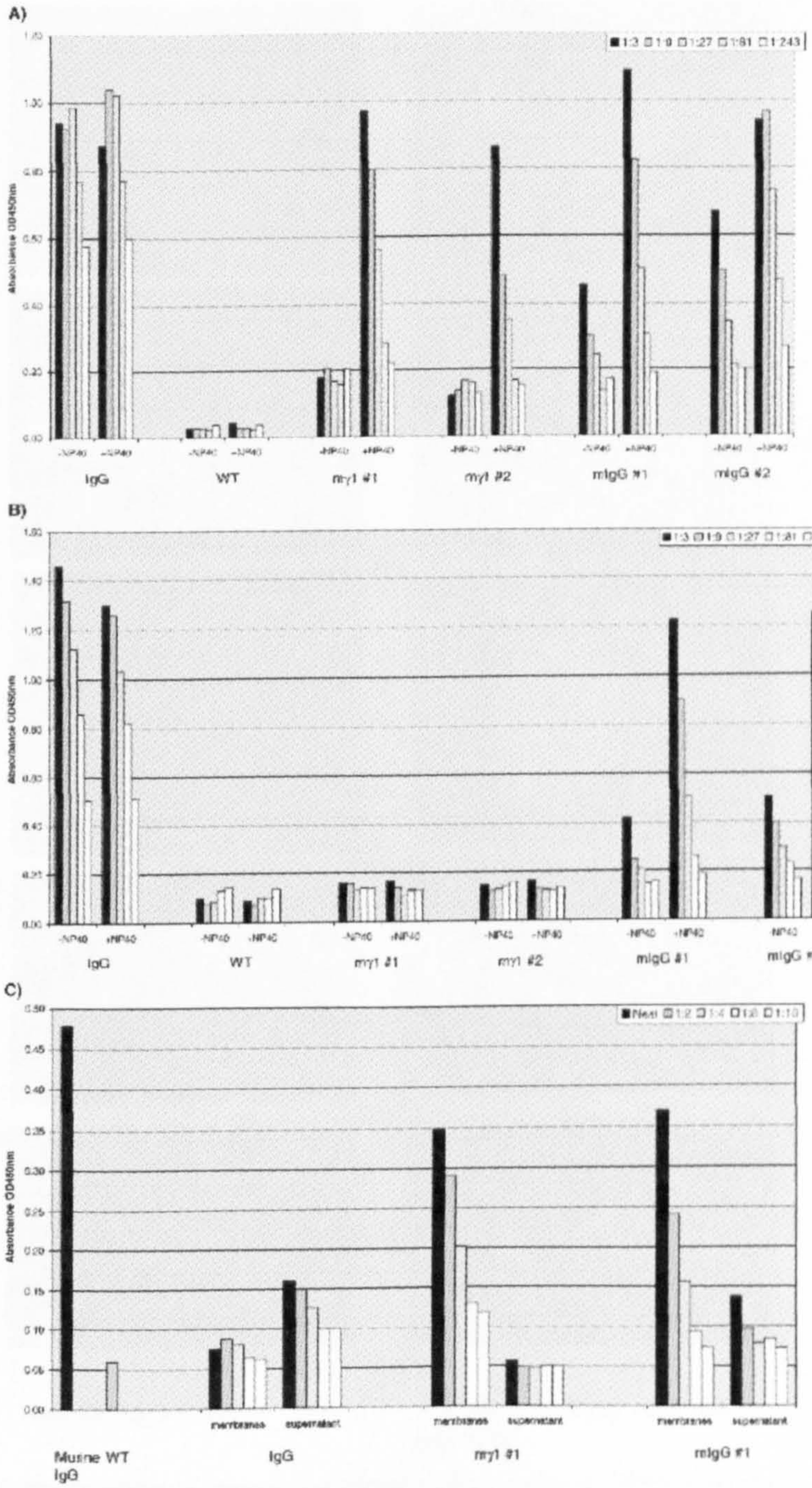

Figure 2. ELISA analysis of plant samples from plants expressing Guy's 13 IgG without transmembrane sequence (IgG), Guy's 13 heavy chain with transmembrane sequence (mγ1) and Guy's mγ1 with Guy's 13 light chain (mIgG). Untransformed plants (WT) were used as a negative control. A. Detection of Guy's 13 γ1 heavy chain by capture ELISA. B. Detection of functional antibody by sandwich ELISA using streptococcal antigen I/II. Extracts were prepared in the presence or absence of non-ionic detergent NP40 as indicated. Titration data are shown, initial sample was a 3-fold dilution of the crude plant extract (black), with subsequent 3-fold dilutions. C. Detection of Guy's 13 mγ1 heavy chain in samples prepared from protoplasts or culture supernatants. The extracts were analysed neat (black) and in 2-fold dilution steps.

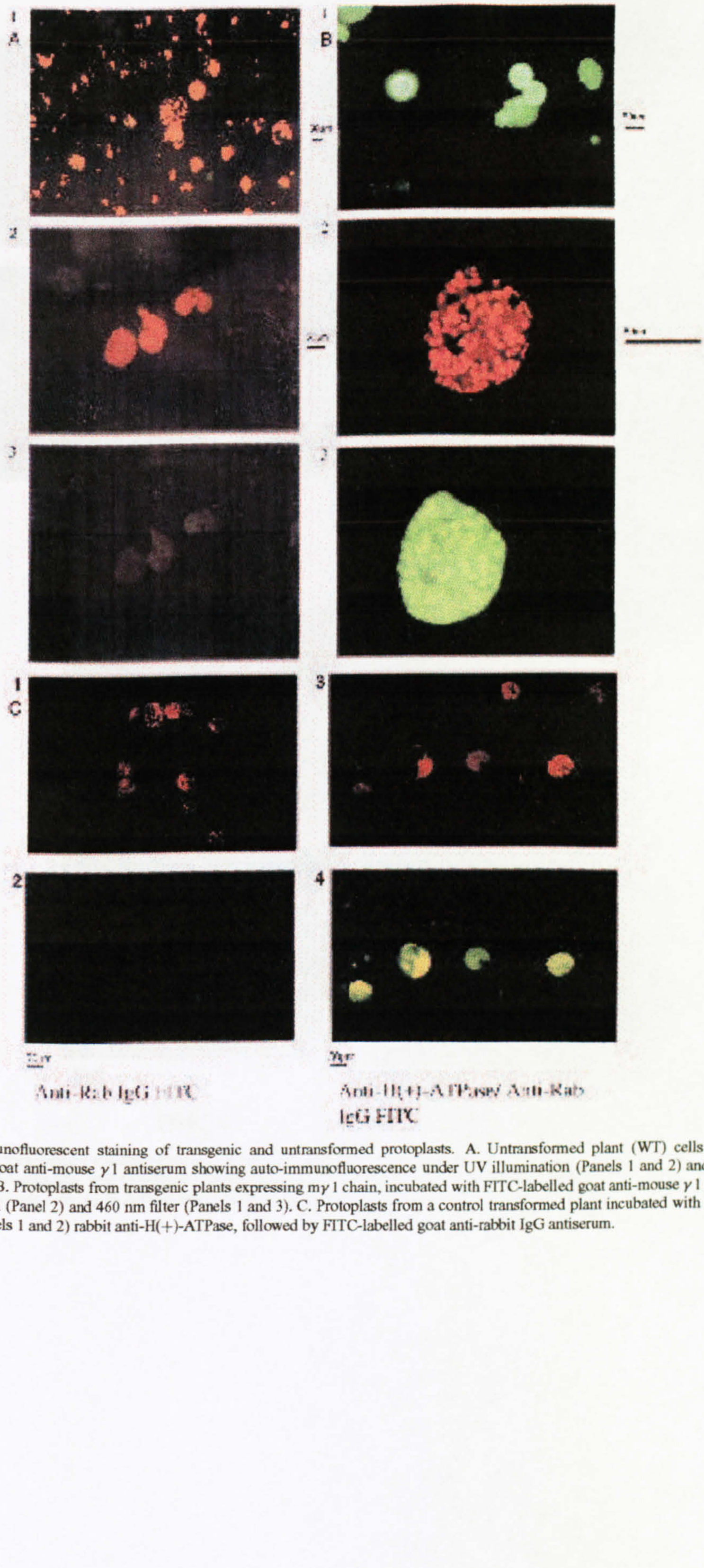

Figure 3. Immunofluorescent staining of transgenic and untransformed protoplasts. A. Untransformed plant (WT) cells incubated with FITC-labelled goat anti-mouse γ1 antiserum showing auto-immunofluorescence under UV illumination (Panels 1 and 2) and with a 460 nm filter (Panel 3). B. Protoplasts from transgenic plants expressing mγ1 chain, incubated with FITC-labelled goat anti-mouse γ1 antiserum under UV illumination (Panel 2) and 460 nm filter (Panels 1 and 3). C. Protoplasts from a control transformed plant incubated with (Panels 3 and 4) or without (Panels 1 and 2) rabbit anti-H(+)-ATPase, followed by FITC-labelled goat anti-rabbit IgG antiserum.

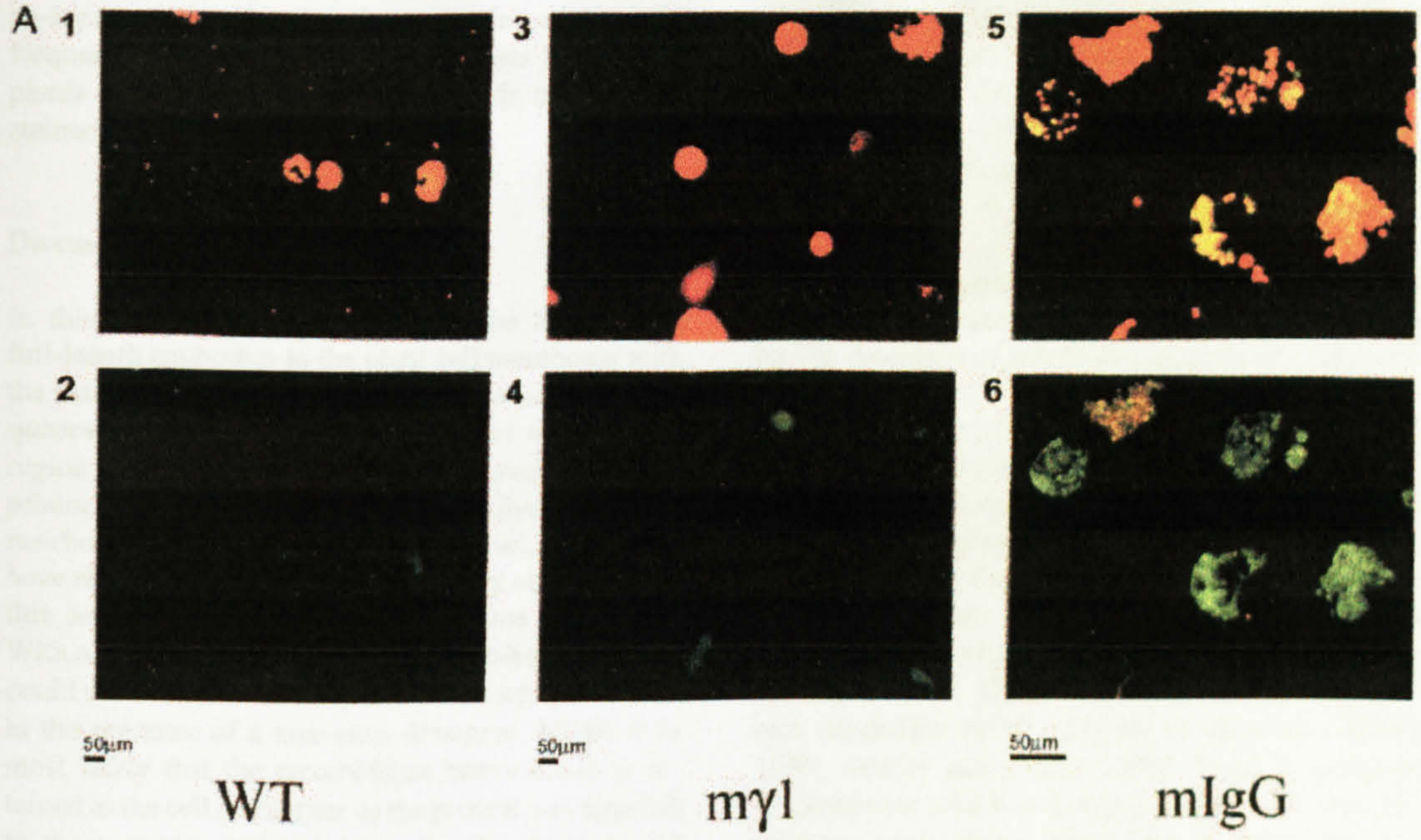

Figure 4. Immunofluorescent staining of transgenic and untransformed protoplasts with FITC-labelled goat anti-mouse antisera. Samples are shown with UV illumination, with or without a 460 nm filter. A. Anti-κ-chain detection: WT cells (Panels 1 and 2), mγ 1cells (Panels 3 and 4) and mIgG cells(Panels 5 and 6). B. Anti-γ-chain detection: mIgG cells (Panels 1 and 2) and mγ 1 cells (Panels 3 and 4).

ulin heavy chain in mIgG plants, as well as the co-localisation with κ chain at the same site. The frequency of brightly stained protoplasts from mIgG plants was similar to that found with mγ1 plants stained with anti-γ-chain antiserum.

Discussion

In this study we have investigated the targeting of full-length antibodies to the plant cell membrane with the native murine immunoglobulin transmembrane sequence. The sequence encodes a short extracellular region, a hydrophobic transmembrane sequence comprising 26 amino acid residues and a hydrophilic 28 residue intracellular domain (Tyler *et al.*, 1982). We have shown that immunoglobulin heavy chain bearing this sequence is retained by the plasma membrane. With a simple procedure, soluble recombinant protein could not be detected unless extraction was performed in the presence of a non-ionic detergent, NP40. It is most likely that the recombinant heavy chain is retained in the cell membrane as the protein was targeted to the secretory pathway by a signal sequence, and also because the heavy chain was readily detected by immunofluorescence of protoplasts. Furthermore, the mγ1 assembled correctly with the immunoglobulin light chain which does not possess a transmembrane sequence but is also targeted for secretion using a related signal sequence. Assembly of light and heavy chain occurs in the ER in plants (manuscript in preparation) and mammals and they emerge at the cell membrane as fully assembled immunoglobulin. We have demonstrated in the mIgG plants that the κ- and γ-chains are co-localised to the cell membrane using immunofluorescence and that they remain correctly assembled at this site as antibody, using a functional antigen-specific ELISA. The staining pattern of both mγ1 and mIgG at the plasma membrane was similar to that seen for a native plasma membrane component (H(+)-ATPase pump), indicating that these recombinant proteins are being targeted to the plasma membrane in a normal manner. Our studies do not however exclude the possibility that the recombinant antibodies might also be distributed elsewhere in the plant cell. Recently it has been demonstrated that engineered immunoglobulins originally targeted for secretion can also be retained in the ER and the vacuole (Frigerio *et al.*, 2000). It remains to be determined if the murine immunoglobulin transmembrane sequence possesses signals that are specifically recognised by plants, but the accumulation of a significant proportion of recombinant immunoglobulin heavy chain and antibody at the cell membrane suggests a default secretory pathway to the cell surface. Further investigation of the fine cellular distribution of mγ1 and mIgG is required, and these molecules may turn out to be useful tools for the study of the default pathway for plant membrane proteins.

The mIgG appears to be more readily dissociated from plant cell membranes than mγ1 alone as shown by the detection of antibodies in plant extracts without NP-40. This may be due to over-accumulation of IgG in the extracellular compartment, or it is possible that the additional burden of the membrane anchored recombinant antibody destabilises the cell membrane. However, protoplasts prepared from these plants appeared no less robust than others prepared from untransformed plants. Not all the protoplasts expressed the mγ1 heavy chain and this is consistent with the finding that the 35S CaMV promoter does not direct expression in all cells all of the time (Barnes, 1990; Benfey and Chua, 1990). Work is going on to determine which cell types express the recombinant immunoglobulin chains and at which stages of development.

There are many potential applications of this form of antibody expression in plants, particularly for disease resistance, in which the retention and accumulation of specific antibodies in an extracellular compartment might help to prevent infection by interfering with microbial invasion. It is unlikely that non-invasive antigens could be taken up across an intact cell wall, but there may also be applications related to the use of cell suspension cultures or protoplasts, for example in large-scale adsorption of antigens. Furthermore, plant antibodies that accumulate but are largely retained in the apoplasm may be useful in the area of phytoremediation and environmental clean-up. The use of this membrane targeting sequence is not confined to immunoglobulins, and other bioactive proteins could be expressed at the cell surface. As a scientific tool this transmembrane sequence could be useful as a marker for analysis of protein expression in plants, as well as having applications in the sorting of protoplasts by fluorescence-activated cell sorting (FACS) (Galbraith, 1989). In mammalian cells, cross-linking of surface proteins often leads to capping and internalisation and if this occurs in plants, cell surface antibodies could be used as a means of internalising foreign molecules into protoplasts.

Acknowledgements

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References

Artsaenko, O., Peisker, M., zur Nieden, U., Fiedler, U., Weiler, E.W., Muntz, K. and Conrad, U. 1995. Expression of a single chain Fv antibody against abscisic acid creates a wilty phenotype in transgenic tobacco. Plant J. 8: 745–750.

Barnes, W.M. 1990. Variable patterns of expression of luciferase in transgenic tobacco leaves. Proc. Natl. Acad. Sci. USA 87: 9183–9187.

Benfey, P.N. and Chua, N.-H. 1990. The cauliflower mosaic virus 35S promoter: combinatorial regulation of transcription in plants. Science 250: 959–966.

Chomczynski, P. and Sacchi, N. 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162: 156–195.

Firek, S., Draper, J., Owen, M.R.L., Gandecha, A., Cockburn, B. and Whitelam, G.C. 1993. Secretion of a functional single-chain Fv protein in transgenic tobacco plants and cell suspension cultures. Plant Mol. Biol. 23: 861–870.

Fraley, R.T., Rogers, S.G., Horsch, R.B., Sanders, P.R., Flick, J.S., Adams, S.P., Bittner, M.L., Brand, L.A., Fink, C.L., Fry, J.S., Galluppi, G.R., Goldberg, S.B., Hoffmann, N.L. and Woo, S.C. 1983. Expression of bacterial genes in plant cells. Proc. Natl. Acad. Sci. USA 80: 4803–4807.

Frigerio, L., Vine, N.D., Pedrazzini, E., Hein, M.B., Wang, F., Ma, J.K.-C. and Vitale, A. 2000. Assembly, secretion and vacuolar delivery of a hybrid immunoglobulin in plants. Plant Physiol. 123: 1483–1493.

Galbraith, D.W. 1989. Analysis of higher plants by flow cytometry and cell sorting. Int. Rev. Cytol. 116: 165–228.

Hein, M.B., Tang, Y., McLeod, D.A., Janda, K.D. and Hiatt, A.C. 1991. Evaluation of immunoglobulins from plant cells. Biotechnol. Prog. 7: 455–461.

Hiatt, A.C., Cafferkey, R. and Bowdish, K. 1989. Production of antibodies in transgenic plants. Nature 342: 76–78.

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G. and Fraley, R.T. 1985. A simple and general method for transferring genes into plants. Science 227: 1229–1231.

Kabat, E.A., Wu, T.T., Perry, H.M., Gottesman, K.S. and Foeller, C. 1999. Sequences of proteins of immunological interest. National Institutes of Health, USA.

Ma, J.K.-C. and Hiatt, K. 1996. Expressing antibodies in plants for immunotherapy. In: M.R.L. Owen and J. Pen (Eds.) Transgenic Plants: A Production System for Industrial and Pharmaceutical Protein, John Wiley, Chichester, UK.

Ma, J.K.-C., Lehner, T., Stabila, P., Fux, C.I. and Hiatt, A. 1994. Assembly of monoclonal antibodies with IgG1 and IgA heavy chain domains in transgenic tobacco plants. Eur. J. Immunol. 24: 131–138.

Owen, M., Gandecha, A., Cockburn, B. and Whitelam, G. 1992. Synthesis of a functional anti-phytochrome single chain Fv protein in transgenic tobacco. Bio/technology 10: 790–794.

Rogers, S.G., Klee, H.J., Horsch, R.B. and Fraley, R.T. 1987. Improved vectors for plant transformation: expression cassette vectors and new selectable markers. Meth. Enzymol. 153: 253–276.

Schouten, A., Roosien, J., van Engelen, F.A., de Jong, G.A.M., Borst-Vrenssen, A.W.M., Zilverentant, J.F., Bosch, D., Stiekema, W.J., Gommers, F.J., Schots, A. and Bakker, J. 1996. The C-terminal KDEL sequence increases the expression level of a single-chain antibody designed to be targeted to both the cytosol and the secretory pathway in transgenic tobacco. Plant Mol. Biol. 30: 781–93.

Smith, R. and Lehner, T. 1989. Characterisation of monoclonal antibodies to common protein epitopes on the cell surface of *Streptococcus mutans* and *Streptococcus sobrinus*. Oral Microbiol. Immunol. 4: 153–158.

Tavladoraki, P., Benvenuto, E., Trinca, S., De Martinis, D., Cattaneo, A. and Galeffi, P. 1993. Transgenic plants expressing a functional single-chain Fv antibody are specifically protected from virus attack. Nature 366: 469–472.

Tyler, B.M., Cowman, A.F., Gerondakis, S.D., Adams, J.M. and Bernard, O. 1982. mRNA for surface immunoglobulin gamma chains encodes a highly conserved transmembrane sequence and a 28 residue intracellular domain. Proc. Natl. Acad. Sci. USA 79: 2008–2112.

Yamawaki-Kataoka, Y., Nakai, S., Miyata, T. and Honjo, T. 1982. Nucleotide sequences of gene segments encoding membrane domains of immunoglobulin gamma chains. Proc. Natl. Acad. Sci. USA 79: 2623–2627.

Assembly, Secretion, and Vacuolar Delivery of a Hybrid Immunoglobulin in Plants

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Secretory immunoglobulin (Ig) A is a decameric Ig composed of four α-heavy chains, four light chains, a joining (J) chain, and a secretory component (SC). The heavy and light chains form two tetrameric Ig molecules that are joined by the J chain and associate with the SC. Expression of a secretory monoclonal antibody in tobacco (*Nicotiana tabacum*) has been described: this molecule (secretory IgA/G [SIgA/G]) was modified by having a hybrid heavy chain sequence consisting of IgG γ-chain domains linked to constant region domains of an IgA α-chain. In tobacco, about 70% of the protein assembles to its final, decameric structure. We show here that SIgA/G assembly and secretion are slow, with only approximately 10% of the newly synthesized molecules being secreted after 24 h and the bulk probably remaining in the endoplasmic reticulum. In addition, a proportion of SIgA/G is delivered to the vacuole as at least partially assembled molecules by a process that is blocked by the membrane traffic inhibitor brefeldin A. Neither the SC nor the J chain are responsible for vacuolar delivery, because IgA/G tetramers have the same fate. The parent IgG tetrameric molecule, containing wild-type γ-heavy chains, is instead secreted rapidly and efficiently. This strongly suggests that intracellular retention and vacuolar delivery of IgA/G is due to the α-domains present in the hybrid α/γ-heavy chains and indicates that the plant secretory system may partially deliver to the vacuole recombinant proteins expected to be secreted.

The secretory pathway delivers proteins from the endoplasmic reticulum (ER), where secretory proteins are cotranslationally inserted, to the cell surface or the inner hydrolytic compartments (vacuoles in plants and yeast, or lysosomes in animals). This traffic is often mediated by the Golgi complex. A vast array of proteins of pharmaceutical importance are secreted by different mammalian cells, and transgenic plants are an attractive expression system for producing recombinant forms of these proteins. One of the major advantages is that many steps of the secretory pathway, including protein folding, assembly, ER-mediated glycosylation, and early steps of Golgi-mediated glycan processing, are largely similar, when not identical, to those found in mammalian cells (Ma and Hein, 1995; Rayon et al., 1998; Sanderfoot and Raikhel, 1999; Vitale and Denecke, 1999). Thus mammalian-secreted proteins can be produced with a high degree of fidelity. One such example is murine immunoglobulin (Ig) G monoclonal antibody. This molecule comprises four polypeptides—two each of a heavy and a light chain, which are linked by disulfide bonds. In mammalian plasma cells, correct assembly of this molecule is achieved within the ER through interactions with a number of chaperones and enzymes and the addition of glycans prior to secretion from the cell. These events are faithfully reproduced in plant cells, to the extent that IgG can be expressed that is functionally indistinguishable from the same antibody expressed in murine cells and is expressed at levels of 1% of total soluble leaf protein (Hiatt et al., 1989; Ma et al., 1995).

When a related but more complex Ig, a recombinant secretory Ig A/G (SIgA/G) hybrid, was expressed in plants, the levels of accumulation were even greater, amounting to 5% to 8% of total soluble leaf protein (Ma et al., 1995). Secretory IgA (SIgA) is a decameric polypeptide complex. In the ER of plasma cells, two standard Ig units (each composed of two heavy and two light chains, like IgG) are first dimerized by a joining (J) chain to form dimeric IgA (dIgA), held by a disulfide bond between each tetramer and the J chain. In mammals, after secretion from plasma cells, dIgA is recognized by a receptor present on the basolateral surface of epithelial cells. Transcytosis causes transport of the receptor/ligand complex to the apical surface, where a proteolytic event releases the dIgA associated to a portion of the receptor (called secretory component [SC]), resulting in the formation of the full, decameric SIgA molecule

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(Mestecky and McGhee, 1987). Thus, in its natural environment SIgA is the result of the activities of two cell types, but in transgenic plants it is successfully synthesized in the ER of individual cells. One of the roles of the SC is to protect the secretory Ig against proteolysis in vivo (Underdown and Dorrington, 1974). Thus, SIgA is a more stable molecule than IgG, however it is unlikely that this alone would account for the difference in accumulation levels between SIgA/G and IgG in plants. In this study we compared the fates of the IgG and SIgA/G in plant cells to determine if there were any differences in the way plants handle these proteins.

One of the peculiar characteristics of the plant secretory pathway is the presence of vacuoles. Although yeast has vacuoles as well and mammals have lysosomes (rich in hydrolases, similar to lytic plant vacuoles), the ontogeny, variety of functions, and protein sorting mechanisms of plant vacuoles are unique (Neuhaus and Rogers, 1998; Marty, 1999). We report that after entry into the plant secretory pathway, a relevant proportion of SIgA/G, but not of IgG, is diverted from secretion and eventually delivered as at least partially assembled molecules to the central vacuole of tobacco (*Nicotiana tabacum*) leaf mesophyll cells. This process is sensitive to the membrane traffic inhibitor brefeldin A. Thus, plant cells may deliver to the vacuole a proportion of mammalian recombinant proteins that are expected to be secreted.

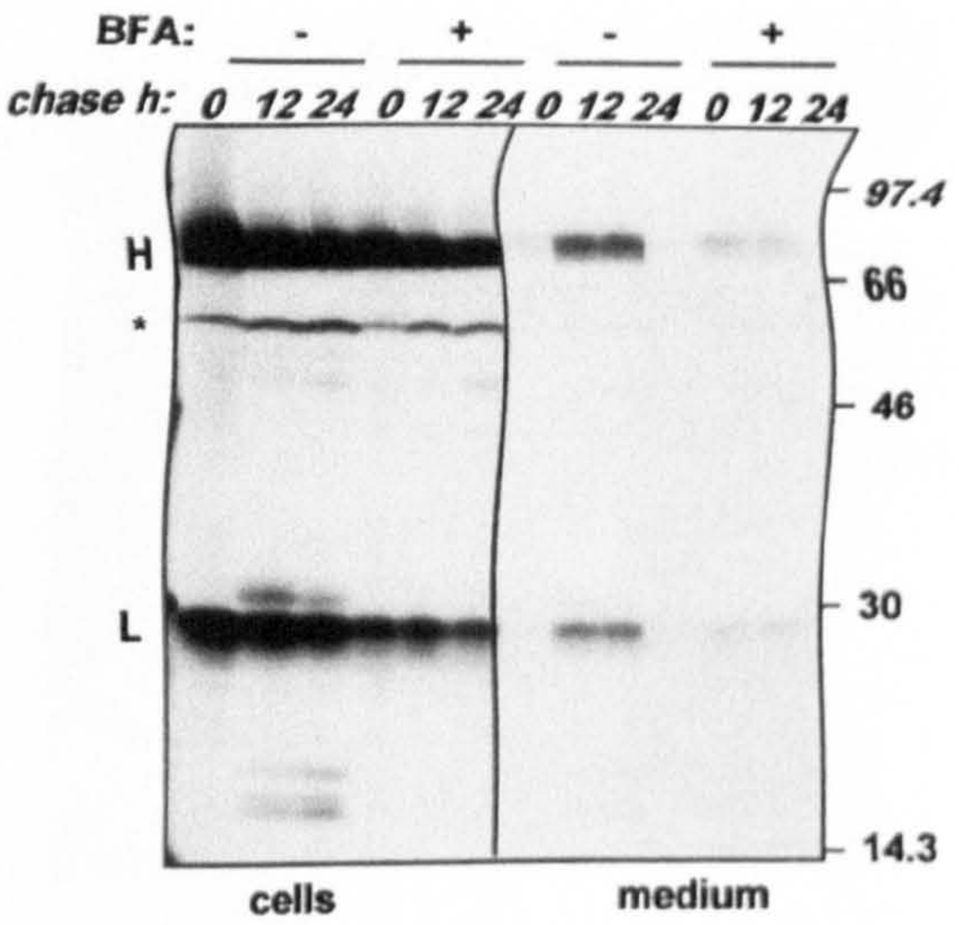

Figure 1. SIgA/G is secreted slowly. Protoplasts from leaves of transgenic tobacco expressing SIgA/G were pulse-labeled for 1 h with [^{35}S]Met and [^{35}S]Cys either in the presence (+) or in the absence (−) of brefeldin A (BFA) and chased for the indicated periods of time. Cells and the corresponding incubation media were homogenized, subjected to immunoprecipitation with anti-IgG antiserum, and analyzed by 15% (w/v) reducing SDS-PAGE and fluorography. Numbers at right indicate molecular mass markers in kD. H, Heavy chain; L, light chain; asterisk, un-glycosylated SC.

RESULTS

Secretion of SIgA/G Is Slow

In SIgA/G, the heavy chains are hybrids containing the variable domains and the constant Cγ1 and Cγ2 domains of the IgG γ-chain linked to the constant Cα2 and Cα3 domains of the IgA α-chain. These extra domains allow the Ig units (composed of two heavy and two light chains) to dimerize by binding the J chain and to further associate with the SC (Ma et al., 1995).

We first wanted to establish the efficiency of SIgA/G secretion. We isolated protoplasts from leaves of SIgA/G-expressing tobacco plants and subjected them to pulse-chase analysis. We then immunoprecipitated the whole SIgA/G molecule from cell lysates and incubation media using polyclonal anti-IgG antiserum, and analyzed the polypeptides on reducing SDS-PAGE and fluorography. Figure 1 shows that, at the end of the pulse period, the heavy IgA/G chain, and the light chain, together with a polypeptide of the expected size for the unglycosylated SC are detected. Glycosylated SC comigrates with the heavy chain, and the J chain, which normally forms dimers on reducing SDS-PAGE, comigrates with the light chain (F. Wang and M.B. Hein, unpublished results). After 12 h, only a small proportion (about 10%) of the antibody is retrieved from the medium; the amount of secreted protein increases only slightly in the following 12 h, whereas the bulk of the antibody remains intracellular. At 5 h of chase the amount of secreted IgA/G is below our detection limit (not shown). Thus, secretion of SIgA/G is a very slow and apparently inefficient process. This seems to be close to the bottom limit of a range observed for heterologous proteins introduced into the plant secretory pathway (Denecke et al., 1990), but a variety of wild-type and recombinant proteins are secreted in a much shorter time, with half-times within 2 to 5 h from the time of synthesis (Hunt and Chrispeels, 1991; Matsuoka and Nakamura, 1991; Frigerio et al., 1998). When protoplasts were incubated in the presence of 10 μg mL^{-1} brefeldin A, secretion was greatly reduced (Fig. 1). This concentration of brefeldin A has been shown to inhibit membrane traffic from the ER (Boevink et al., 1998).

After 12 h of chase, several discrete polypeptides in the 15- to 30-kD range were detected in the protoplast lysates (Fig. 1, no brefeldin A treatment). The presence of intracellular Ig breakdown products was previously reported (Ma et al., 1994, 1995). Their absence at the end of the pulse and appearance during the chase rule out the possibility that these fragments were generated during sample homogenation. The appearance of these fragments was efficiently inhibited by brefeldin A treatment (Fig. 1; 12- and 24-h chase in the presence of brefeldin A), indicating that fragmentation occurs because of transit to a post-ER compartment through membrane traffic (see further below).

The Intracellular Breakdown Products Are Located in the Vacuole

We wanted to investigate where the Ig breakdown products are located. The fact that degradation can be prevented by brefeldin A treatment, which inhibits delivery to plant vacuoles (Gomez and Chrispeels, 1993), suggested that the vacuole could be the site of degradation. To test this hypothesis, we subjected SIgA/G-expressing protoplasts to pulse-chase either in the presence or in the absence of brefeldin A. We then purified vacuoles from protoplasts and immunoselected the Ig (Fig. 2). After 16 h of chase the Ig fragmentation products were clearly detectable in the vacuolar fraction; consistently, treatment with brefeldin A prevented the appearance of the fragments in the vacuoles. We then conclude that a proportion of the SIgA/G molecules is diverted to the vacuole by membrane traffic. Intact light chains also seem to be in part located in the vacuoles; whereas the extremely low proportion of intact heavy chains in the vacuolar fraction is most likely a slight contamination from another cellular compartment, because it is also detectable, at similar levels, at the end of the pulse-labeling and is not affected by brefeldin A treatment.

Immunoelectron microscopy indicated that the steady-state level of recombinant protein present in vacuoles was not irrelevant. In sections of leaves from SIgA/G-expressing plants, gold-conjugated anti-γ-chain (small particles) decorated large vacuolar aggregates, which were not detectable in wild-type plants (Fig. 3). These aggregates closely resemble protein body-like structures, which have been

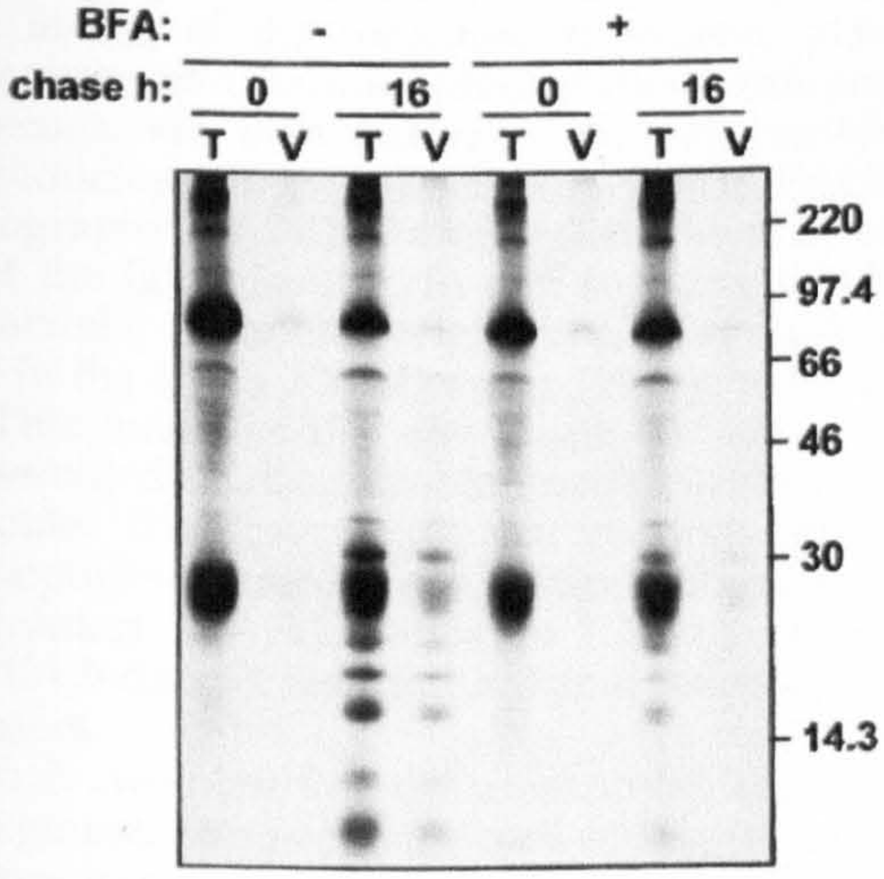

Figure 2. A proportion of SIgA/G is targeted to the vacuole and fragmented. Protoplasts from SIgA/G-expressing plants were pulse-labeled for 1 h either in the presence (+) or in the absence (−) of brefeldin A and chased for the indicated periods of time. Total cell homogenates (T) or purified vacuoles (V) were then subjected to immunoprecipitation with anti-IgG antiserum and analyzed by 15% (w/v) reducing SDS-PAGE and fluorography. Numbers at right indicate molecular mass markers in kD.

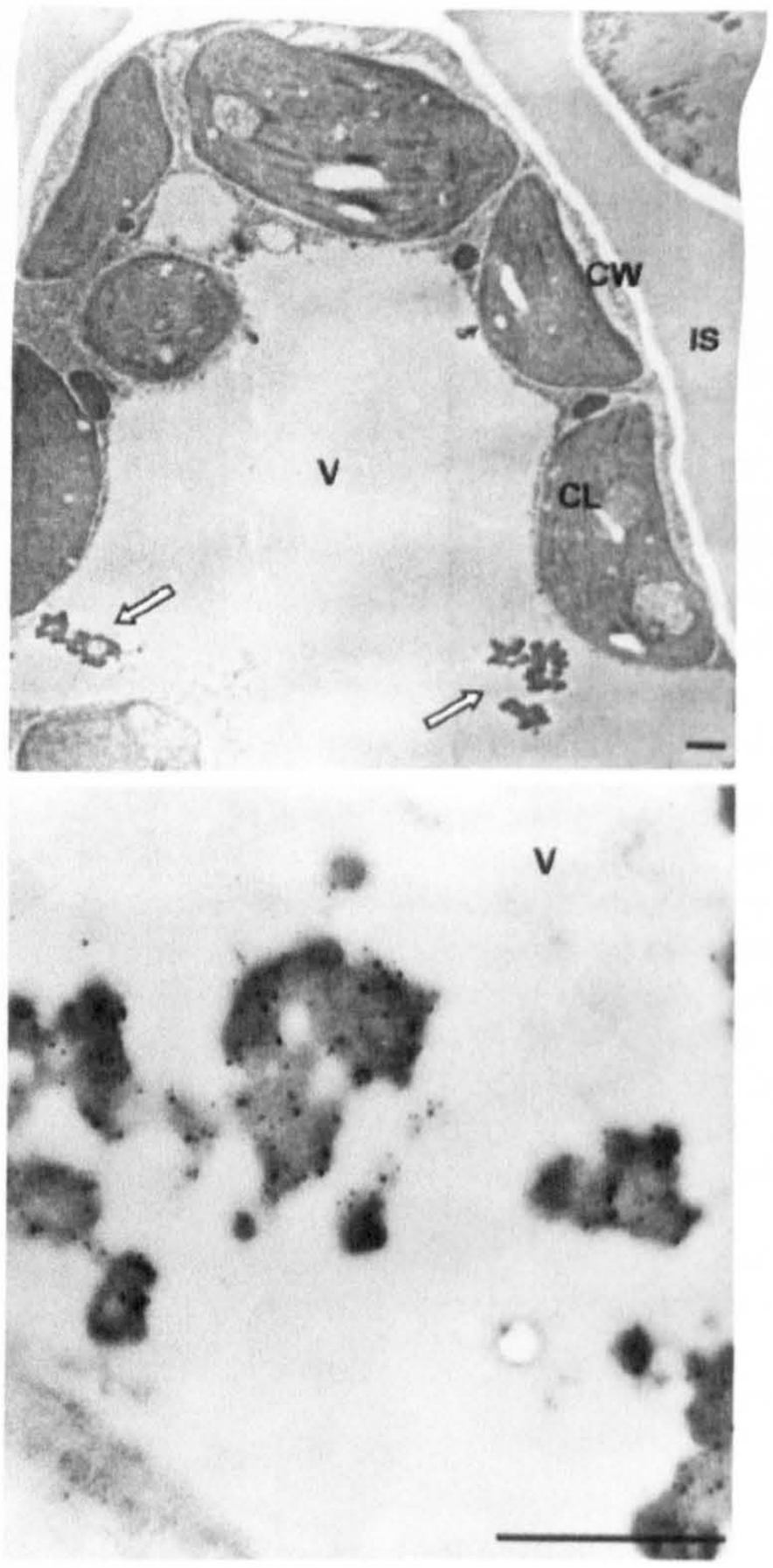

Figure 3. SIgA/G forms protein body-like aggregates in the vacuole. Ultra-thin sections of transgenic tobacco leaves expressing SIgA/G (top panel) were incubated with anti-SC and anti-Ig antibodies (bottom panel). Detection was performed using secondary antibodies conjugated with 12 nm (for SC) and 6 nm (for Ig) colloidal gold. Arrows indicate the vacuolar protein body-like structures. CL, Chloroplast; CW, cell wall; IS, intercellular space; V, vacuole. Bars = 1 μm.

observed when vacuolar proteins (such as barley lectin and bean phaseolin) were expressed in leaf cells (Dombrowski et al., 1993; Frigerio et al., 1998). The anti-SC antibody (large particles) decorated the same protein bodies as the anti-γ chain.

Vacuolar Delivery Is Caused by Sequences in the IgA/G Tetramer

The finding that a percentage of antibody in the SIgA/G plants is not secreted but rerouted to the vacuole, raises the question of whether this vacuolar targeting is the product of a positive signaling by the molecule, or rather represents an escape route for the plant endomembrane system when overloaded with foreign molecules.

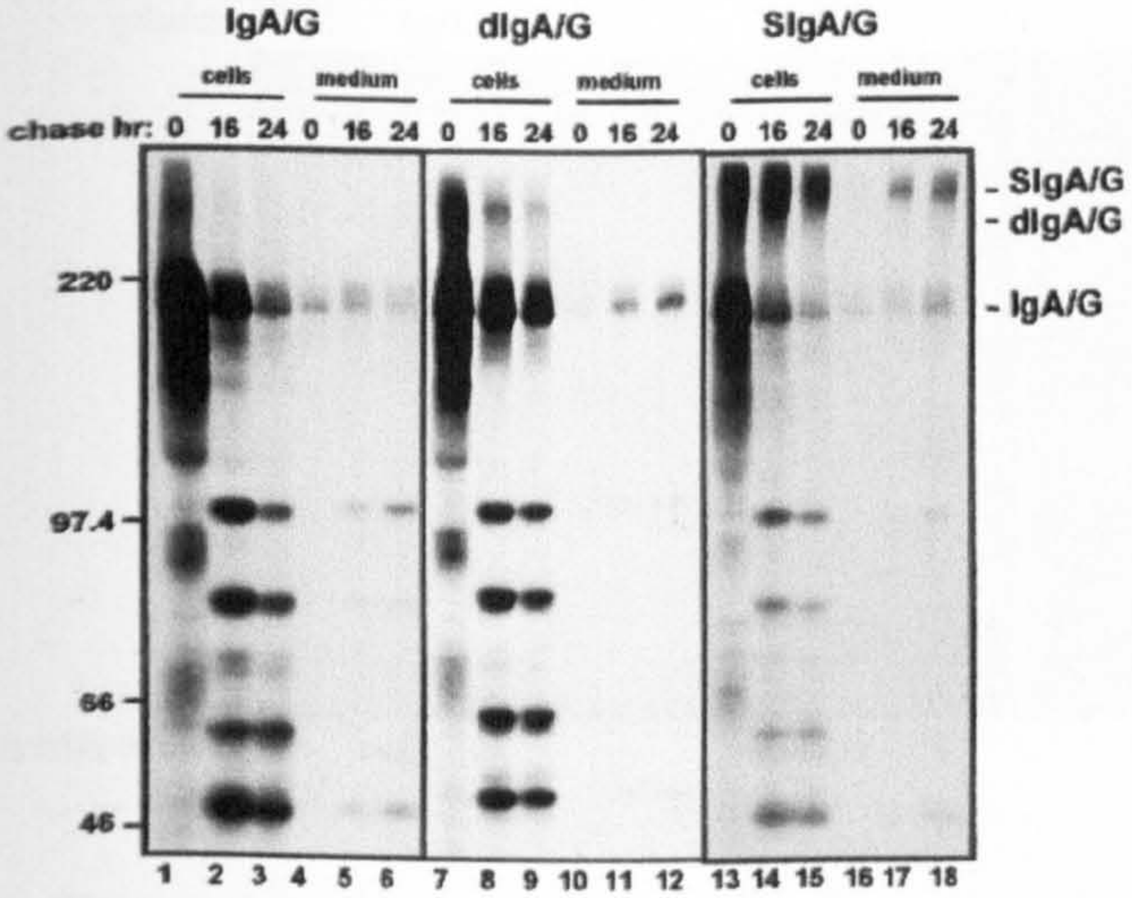

Figure 4. Vacuolar targeting and slow secretion are independent of the assembly state of SIgA/G. Protoplasts from leaves of transgenic tobacco plants expressing IgA/G, dIgA/G, or SIgA/G were pulse-labeled for 1 h and chased for the indicated periods of time. Cells and the corresponding incubation media were homogenated, subjected to immunoprecipitation with anti-IgG antiserum, and analyzed by 6% (w/v) non-reducing SDS-PAGE and fluorography. Numbers at left indicate molecular mass markers in kD.

We first determined whether vacuolar sorting could be caused by one or more components of the SIgA/G molecule, namely the SC or the J chain. Therefore we analyzed transgenic plants expressing either the sole IgA/G tetrameric unit (IgA/G) or the J-chain linked IgA/G dimer (dIgA/G) and compared the fate of these assembly intermediates to that of the whole SIgA/G molecule. We subjected protoplasts from leaves of the indicated transgenic plants to pulse-chase and immunoprecipitation with anti-IgG antiserum. We then analyzed the polypeptides by non-reducing, 6% (w/v) acrylamide SDS PAGE and fluorography (Fig. 4) to establish the level of assembly of the Ig molecules. In non-reducing conditions the vacuolar fragments originating from SIgA/G migrate in the 45- to 100-kD range (Fig. 4, lanes 14 and 15). This indicates that the fragments are not fully unassembled or disassembled components of the Ig molecule. The observation that the majority of the polypeptides migrate under non-reducing conditions as tetrameric IgA/G units after 1 h of pulse (Fig. 4, lane 13) indicates fast and efficient assembly of the tetramers.

Clearly, we observed the presence of identical major fragmentation products in all cell types, including the ones expressing the tetrameric IgA/G unit only (Fig. 4, compare lanes 2 and 3, 8 and 9, 14 and 15). This indicates that delivery to the vacuole does not require the J chain or the SC. The ratio of intact to fragmented molecules was higher in SIgA/G-expressing plants (Fig. 4, lanes 13–18), indicating that either the fully assembled SIgA/G is more resistant to proteolysis in the vacuole or the SC and the J chain partially inhibit vacuolar delivery of IgA/G. The IgA/G unit seemed to be partially degraded even after secretion in the incubation medium (Fig. 4, lanes 5 and 6). It has been reported that proteins secreted by tobacco protoplasts (Frigerio et al., 1998) and cultured cells (Matsuoka et al., 1995) may undergo proteolysis. Again, secretion of SIgA/G was slow and inefficient (Fig. 4, lanes 16–18, and see Fig. 1, lanes 7–9). A similar rate of secretion was observed for the "monomeric" form of the antibody both in IgA/G and dIgA/G plants. No secretion of the dimeric molecule was detected, because either the dimers are unavailable for secretion for unknown reasons or the J chain is particularly susceptible to extracellular proteolysis when not protected by the SC.

The Parent IgG Is Rapidly and Efficiently Secreted

The results shown so far indicate that, even in their simplest, tetrameric form, plant-made IgA/Gs are inefficiently secreted and in part delivered to the vacuole. Is intracellular retention due to domains in the IgA/G molecule or is it due to a more general problem, as the plant secretory system must deal with the synthesis and assembly of complex, bulky molecules such as Igs? If the latter is true, we would expect the parent murine IgG molecule to undergo the same fate as its IgA/G derivative. Indeed, the substitution of the native $C\gamma3$ domain with $C\alpha2$ and $C\alpha3$ domains from an IgA-secreting hybridoma has no negative effect on the assembly and activity of the hybrid molecule (Ma et al., 1995, 1998) and the sequence of assembly events is virtually identical.

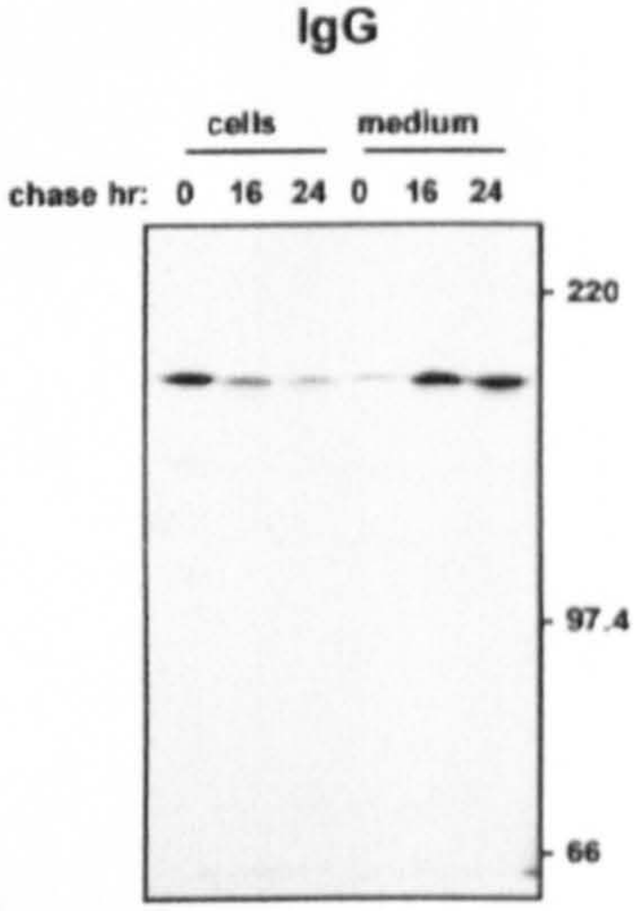

Figure 5. The parent IgG molecule is secreted efficiently. Protoplasts from leaves of transgenic tobacco plants expressing the monoclonal Guy's 13 IgG were pulse-labeled for 1 h and chased for the indicated periods of time. Cells and the corresponding incubation media were homogenized, subjected to immunoprecipitation with anti-IgG antiserum, and analyzed by 6% (w/v) non-reducing SDS-PAGE and fluorography. Numbers at right indicate molecular mass markers in kD.

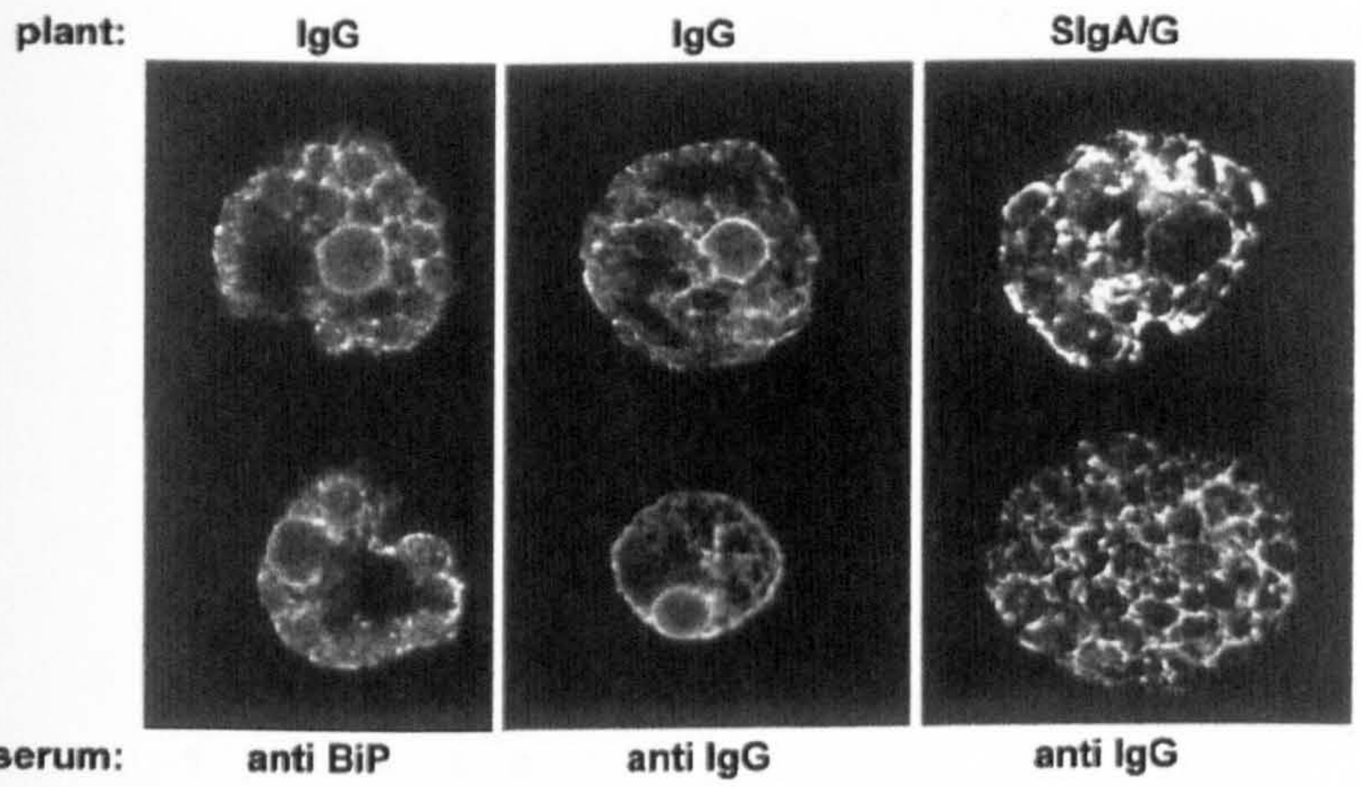

Figure 6. The bulk of SIgA/G is retained in the endomembrane system. Protoplasts from either IgG- or SIgA/G-expressing plants were fixed, permeabilized, and subjected to immunofluorescence with primary rabbit anti-IgG serum or anti-BiP serum, followed by secondary fluorescein isothiocyanate-conjugated goat anti-rabbit antibody. Cells were observed with a confocal laser scanning microscope at 494-nm excitation and 522-nm emission wavelength. For IgG cells, laser intensity was 10%, gain 1,200; for SIgA/G cells, laser intensity was 3%, gain 950.

To test this, we isolated protoplasts from leaves of Guy's 13 IgG producing plants and followed the fate of the IgG molecule by pulse-chase (Fig. 5). We found that almost all the IgG synthesized during the pulse was recovered after 24 h of chase from the protoplast incubation medium. The secreted IgG was tetrameric and intact, and no intracellular degradation products were detectable (Fig. 5). Thus, secretion was nearly quantitative. Moreover, the rate of secretion was much faster than that of SIgA/G: About 70% of the molecules were secreted after 16 h.

The difference in the fate of IgG compared to that of SIgA/G is also evident at steady state (Fig. 6). We detected the Ig molecules by immunofluorescence confocal laser scanning microscopy on fixed, permeabilized protoplasts from IgG or SIgA/G transgenic plants. The anti-IgG antiserum did not stain any structure in cells prepared from un-transformed tobacco (not shown). In the case of IgG-producing cells (Fig. 6, center panel), the steady-state amount of Ig detected in the secretory system represents only the newly synthesized molecules and the molecules en route to secretion, as testified by the relevant proportion of staining in proximity of the plasma membrane. This was confirmed by comparison with the distribution of the ER resident chaperone BiP (Fig. 6, left panel). Whereas the perinuclear and reticular structures were similarly stained, staining in proximity of the plasma membrane was much less intense. It is also evident that the endomembrane system of SIgA/G producing plants is literally laden with antibody molecules (Fig. 6, right panel). The reticular structure is similar to the one detectable using antibodies against the BiP, but in the case of SIgA/G its appearance is more swollen and punctate structures, possibly representing vacuolar depositions, are often detectable as well.

Vacuolar delivery could in theory result from endocytosis of secreted molecules. However, the results shown in Figure 5 indicate that secreted IgGs are certainly not subjected to endocytosis to a relevant extent, ruling out unspecific uptake of protein from the medium. Moreover, when incubation during the chase was performed under agitation in a 10-fold excess of incubation medium with respect to our standard protocol, there was no reduction in the efficiency of IgA/G fragmentation (Fig. 7). Therefore, most probably vacuolar IgA/G is not a fraction of secreted molecules that have been endocytosed.

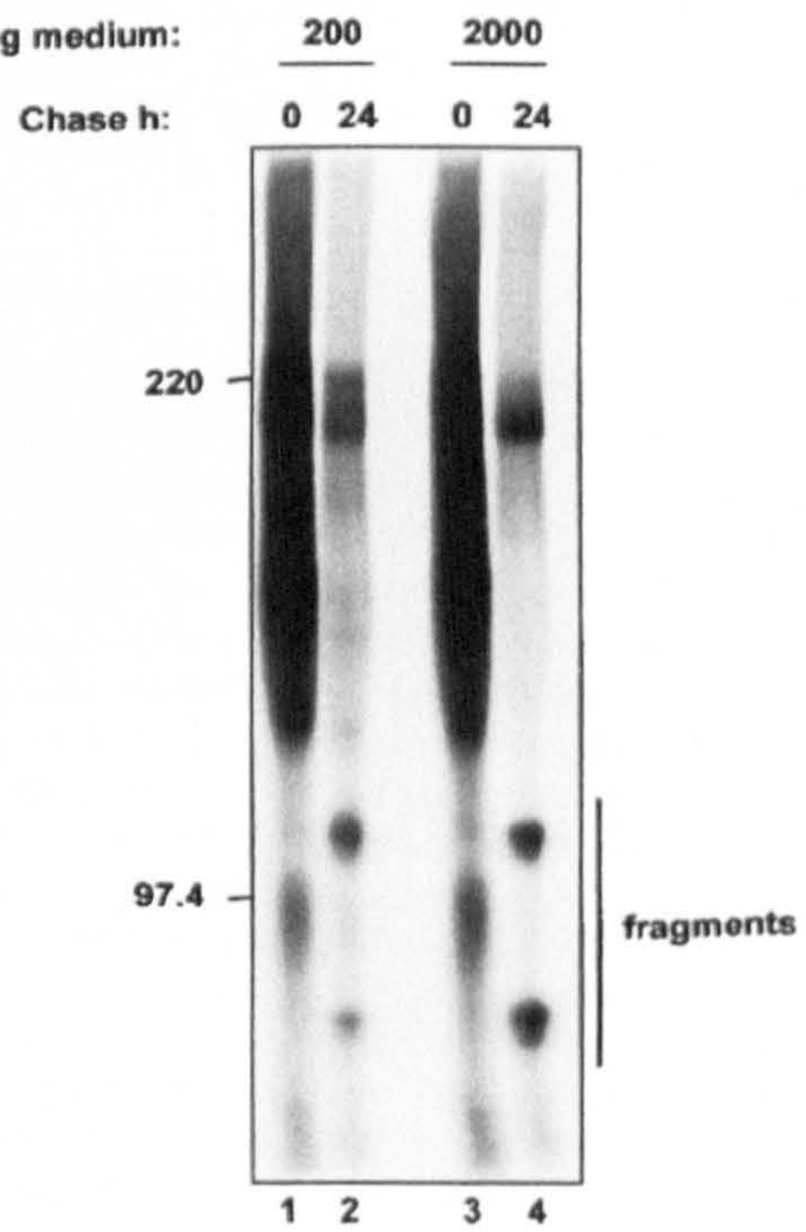

Figure 7. IgA/G fragmentation is not due to endocytosis. Protoplasts from plants expressing IgA/G were pulse-labeled for 1 h in the indicated volumes of incubation medium and chased for the indicated periods of time. Cells were then homogenated and subjected to immunoprecipitation with anti-IgG antiserum, followed by 6% (w/v) non-reducing SDS-PAGE and fluorography. Numbers on the left indicate molecular mass markers in kD. Only the intact protein and the higher molecular mass fragments are shown.

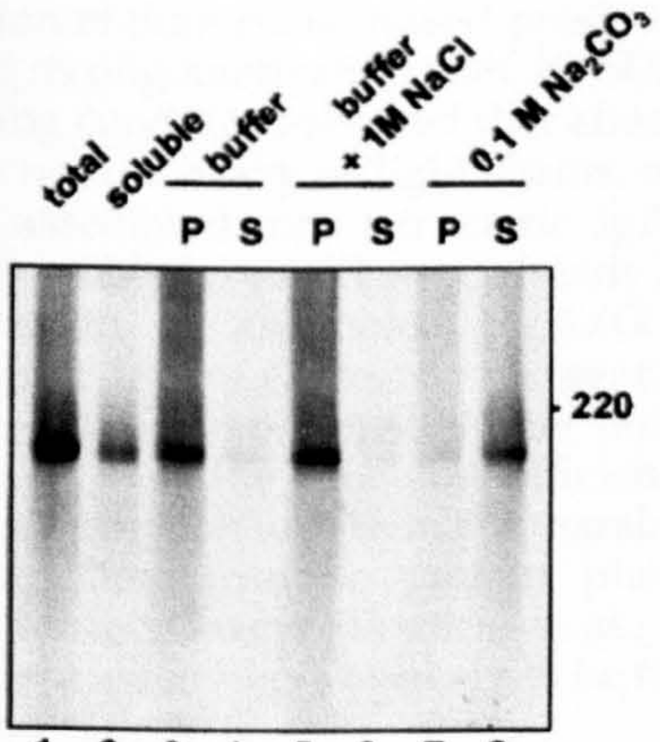

Figure 8. IgA/G does not associate tightly with membranes. Protoplasts from plants expressing IgA/G were pulse-labeled for 1 h and homogenized in 12% (w/w) Suc buffer. Microsomes were prepared and resuspended in Suc buffer, in Suc buffer with 1 M NaCl, or in 0.1 M Na_2CO_3, and incubated on ice for 30 min. Microsomes were then reloaded on top of a 17% (w/w) Suc pad and centrifuged for 30 min at 150,000*g*. Supernatants (S) and pellets (P) were homogenated, immunoprecipitated with anti-IgG antiserum, and analyzed by 6% (w/v) non-reducing SDS-PAGE and fluorography. total, Anti-IgG immunoprecipitate from total cell homogenate. soluble, Anti-IgG immunoprecipitate from the supernatant from the first microsome preparation, containing cytosolic and vacuolar proteins.

Newly Synthesized IgA/G Is a Soluble Protein

In mammalian cells, proteins that are anchored to the ER membrane follow the secretory pathway until they reach the plasma membrane, unless they have specific features that allow their ER retention or sorting to other membranes of the endomembrane system (Pedrazzini et al., 1996). The question of a default location for membrane proteins is still open for plant cells, and the tonoplast is a possible candidate (Barrieu and Chrispeels, 1999). In addition the newly synthesized precursor of the soluble vacuolar storage protein legumin is tightly associated to the lumenal side of ER/Golgi membranes and its membrane binding is unaffected by agents that usually release peripheral membrane proteins (Hinz et al., 1997). It has been suggested that high affinity to membranes may have a role in the vacuolar sorting of legumin and maybe other storage proteins (Saalbach et al., 1991). We therefore verified whether newly synthesized IgA/G is tightly associated to the endomembranes. Protoplasts from transgenic leaves were pulse-labeled for 1 h and homogenated in the absence of detergent and the presence of Suc. The homogenate was fractionated into a microsomal and a soluble fraction. The latter contains cytosolic proteins and the content of vacuoles, which break during homogenation under these conditions. Consistently, newly synthesized IgA/G is mainly recovered in the microsomal fraction (Fig. 8, lanes 1 and 2). Microsomes were then washed with Suc buffer as a control, with 1 M sodium chloride in Suc buffer, or with 0.1 M sodium carbonate. Sodium carbonate releases soluble proteins present in the lumen of microsomes but not integral membrane proteins (Fujiki et al., 1982), whereas sodium chloride releases proteins peripherally attached to the cytosolic face of microsomes without affecting their lumenal content. As expected for a soluble lumenal protein that is not tightly bound to the membrane, IgA/G is released by sodium carbonate but not by sodium chloride (Fig. 8, lanes 3–8). We conclude that tight association to the endomembranes cannot explain the vacuolar delivery of IgA/G.

DISCUSSION

In this paper we show that secretion of SIgA/G proceeds at a very slow rate in tobacco leaf cells. Even 24 h after synthesis, the vast majority of molecules has not been secreted. Moreover, part of the protein is transported to the vacuole where it is detectable as fragmentation products. In contrast the parent IgG molecules are quantitatively secreted with a half-time of less than 16 h. This raises the problem of which are the mechanisms and recognition events that lead to vacuolar delivery of a protein that is expected to be secreted. Besides the implications for the production of IgA in plants, this can shed light on still unknown features of the plant secretory pathway which may have general relevance for both plant metabolism and the use of plants to produce heterologous secretory proteins.

Slow and Inefficient Secretion

Our experiments rule out the possibility that the J chain and the SC are responsible for the slow secretion and for vacuolar delivery of SIgA/G. Therefore, the modification of the heavy chain by deletion of the Cγ3 domain and the addition of Cα2 and Cα3 domains (Ma et al., 1994) is the structural characteristic that leads to the altered secretory phenotype.

The rates of secretion from tobacco protoplasts of three bacterial proteins introduced into the secretory pathway have been compared previously (Denecke et al., 1990). Although the assay used to measure secretion was different from ours, the results pointed to a high degree of variability in secretion efficiency of heterologous proteins in plant cells. An inverse correlation was found in that case between secretory rate and size of the passenger protein, but the reasons for such variability remained unknown.

Secretion rate and efficiency can certainly be determined by ER quality control. This mechanism assists the folding and assembly of newly synthesized proteins, in most cases allows their traffic along the secretory pathway only upon correct completion of these maturation events and eventually targets defective polypeptides for degradation (Vitale and Denecke, 1999). Confocal microscopy suggested that a

large fraction of SigA/G is indeed present in the ER. Analysis of its oligomerization state by SDS-PAGE in non-reducing conditions showed that after 1-h pulse, there were no free heavy or light chains, most of the molecules assembled into tetrameric IgA/G units, and fully assembled SigA/G were already detectable. The proportion of assembled SigA/G increased markedly after 16 h of chase, to represent more than 60% of the total immunoprecipitable polypeptides. This indicates that the rate and efficiency of heterotetramer formation are high, comparable to those of the bean homotrimeric protein phaseolin expressed in tobacco leaves (Frigerio et al., 1998), and that inefficient assembly is unlikely to be the cause of ER retention of IgA/G.

In mammalian cells, exposed Cys residues in the C-terminal region of the heavy chains of IgA and secretory IgM are recognized by ER quality control; as a result, in B lymphocytes tetramers are retained in this compartment (Sitia et al., 1990; Guenzi et al., 1994). The efficiency of this retention is both dependent on the stage of B cell development and the amino acid context surrounding the Cys. In plasma cells, IgA but not IgM are secreted, albeit slowly, and the different behavior of the two Igs is due to the presence of an extra acidic residue upstream of the Cys in IgA but not IgM (Guenzi et al., 1994). On the other hand, mutation of the critical Cys results in very efficient secretion of IgM monomers (Sitia et al., 1990). Thus, in mammalian cells thiol-mediated retention is responsible for the decreasing rates of secretion of IgG, IgA, and secretory IgM tetramers, respectively. Our observation that in tobacco the parent IgG tetramers, which do not have free cysteines, are secreted with high efficiency is consistent with the possibility that thiol-mediated retention also occurs in the plant ER. This hypothesis can be tested by mutagenesis or in vivo treatment with reducing agents.

We cannot rule out the alternative possibility that, in spite of correct assembly and independently of the exposed Cys residues, some of the tetramers or decamers have conformational defects that lead to their prolonged ER retention and eventual slow degradation. The IgA/G heavy chain is not a naturally synthesized molecule—it contains a mixture of γ- and α-chain domains, as well as an extra Cα2 domain. Although this might affect the dimerization of heavy chains, it seems unlikely that this modification would affect assembly with Ig light chain, or even J chain and SC. However, we have no experience of how addition of an extra Cα2 domain in the heavy chain might affect interactions with chaperones or recognition by quality control mechanisms in the ER.

Delivery to the Vacuole

Fragmentation of a proportion of the IgA/G (monomer, dimer, or secretory) molecules occurs either soon before or upon delivery to the vacuole: Fragments are detected in vacuoles. This vacuolar delivery is inhibited by brefeldin A, indicating active vesicular transport out of the ER along the secretory pathway.

Plant cells, like mammalian cells, have a default route that delivers out of the cell proteins that are inserted into the ER (Denecke et al., 1990). To be delivered to the vacuole, soluble proteins need sorting signals; when these signals are deleted, the mutated proteins are secreted, albeit with variable efficiencies (Bednarek et al., 1990; Crofts et al., 1999). Although one potential receptor for vacuolar sorting has been identified, the mechanisms for vacuolar delivery are not yet fully clarified, and certainly more than one mechanism exists (Vitale and Raikhel, 1999). It has been hypothesized that aggregation and high affinity to membranes can be the mechanism that sorts some storage proteins to vacuoles (Saalbach et al., 1991; Vitale and Raikhel, 1999). However we were unable to demonstrate tight binding of IgA/G to endomembranes. Endocytosis followed by vacuolar delivery also seems an unlikely possibility in the light of our results.

We are not aware of previous reports of vacuolar delivery of a protein expected to be secreted in plant cells, but other proteins that do not reside in the vacuole in their natural cells are in part delivered to vacuoles when expressed in transgenic plants. These are the maize zeins, which in maize accumulate as ER-located protein bodies but are partially found in vacuoles in transgenic tobacco (Coleman et al., 1996). The recognition events that lead to this mislocalization have not been established, but autophagy has been proposed as a possible explanation (Coleman et al., 1996). It is not known whether this process can be inhibited by brefeldin A.

In mammalian cells, prolonged ER retention of unpolymerized IgA and IgM tetramers or certain unassembled mutant light chains can result in quality control degradation. Degradation of unassembled light chains, IgM and J chains is sensitive to proteasome inhibitors, strongly suggesting that it occurs upon dislocation of the polypeptides from the ER into the cytosol (Chillaron and Haas, 2000; Mancini et al., 2000; C. Fagioli and R. Sitia, personal communication), a fate similar to the one of several other mammalian and yeast proteins subjected to ER associated degradation (Brodsky and McCracken, 1999). Therefore, if quality control is responsible for the fragmentation of IgA/G in tobacco, the subcellular location of the hydrolytic activity is different with respect to the one identified in mammalian cells. In yeast cells, it has been shown that defective proteins can be delivered for degradation to the vacuole through the Golgi complex (Hong et al., 1996); again the recognition mechanism is not clear but this has been suggested to be a quality control disposal route alternative to retrotranslocation from the ER in the

cytosol, which conversely does not involve vesicular traffic. Such a Golgi-mediated route may be active also in plant cells and take care of the degradation of a proportion of antibody molecules with exposed Cys residues or defects that could be not be detected by our assays.

The polypeptides targeted to the vacuole and fragmented are, at least in part, assembled, although we have not yet determined their exact assembly status. In the light of the analysis of the oligomerization state after pulse-labeling, the most reasonable hypothesis is that the major Ig form delivered to the vacuole is the tetrameric unit. A proportion of these units would be delivered to the vacuole after prolonged ER retention. The colocalization of the heavy chain and the SC in the same vacuolar aggregates, detectable by immunoelectron microscopy, does not necessarily imply that the two molecules are part of the same assembled antibody: when two noninteracting vacuolar proteins were co-expressed in transgenic plants they colocalized in vacuolar aggregates as well (Schroeder et al., 1993). The proportion of SC found in the vacuole could in this case be delivered to this compartment in different forms: associated to intermediates of assembly or as individual, unassembled molecules, whereas fully assembled SIgA/G would be secreted.

An alternative hypothesis with respect to quality control delivery to the vacuole could be that saturation of secretion, due to high levels of Ig synthesis, leads to delivery of the excess of protein to the vacuole, independently of the presence of the J chain and SC. We do not favor this hypothesis, because of the behavior of IgG and of the much higher proportion of fragmented IgA/G molecules generated in the absence of the J chain and the SC, but it cannot be conclusively ruled out. The opposite is certainly true: Overexpression of vacuolar proteins leads to their partial Golgi-mediated secretion (Frigerio et al., 1998). Vacuolar delivery upon high expression would imply that secretion from plant cells is a saturable process. Such a scenario would be against the current model that secretion is the default route for proteins inserted into the secretory pathway. There is very solid evidence that a default route to secretion exists, but several observations suggest that active selection of cargo proteins destined for secretion may also occur (Vitale and Denecke, 1999). The two mechanisms, default and active selection, might co-exist, the latter being saturable and its saturation leading to vacuolar delivery as an alternative route. This hypothesis can be tested by co-expressing with SIgA/G a protein known to be secreted by plant cells with high efficiency.

Finally, irrespective of the quality control or saturation hypothesis, our observation that vacuolar fragmentation is higher in the absence of the SC, also when the J chain that allows dimerization is synthesized, indicates an active role for the SC in promoting secretion and/or protecting from degradation in the vacuole. We believe this is important. It should be remembered that in the natural organism where SIgA are produced, addition of the SC is an extracellular event. Clearly, this component is also able to alter the destiny of Ig molecules within the plant endomembrane system.

Localization and Stability

SIgA/G and the parent IgG accumulate in tobacco leaves to 5% to 8% and 1% of total soluble proteins, respectively (Ma et al., 1994, 1995). What is the reason of this difference? We originally hypothesized that the SC might protect SIgA/G from degradation in the apoplast (Ma et al., 1995). The results presented here suggest other possible explanations.

Certainly, a proportion of SIgA/G is present in the ER and in the vacuole. The ER has been shown to be a very safe compartment for recombinant secretory proteins expressed in transgenic plants: when proteins were retained in the ER via the addition of the KDEL signal they showed markedly increased stability (Wandelt et al., 1992; Pueyo et al., 1995; Tabe et al., 1995; Conrad and Fiedler, 1998). ER retention of SIgA/G is certainly not due to the presence of a known ER retention signal, and might instead be due to quality control, which in this case could favor stability. In transgenic tobacco, a mutated phaseolin form, that is unable to assemble and is much more susceptible to in vitro proteolysis than wild-type phaseolin, is subjected to prolonged retention in the ER by quality control and accumulates to levels comparable to the wild type counterpart (Pedrazzini et al., 1997).

It is also possible that part of the SIgA/G fragments present in the vacuole retain their activity, although most probably complete degradation of Ig occurs therein to some extent. In this respect, solving the problem that leads to partial vacuolar delivery of SIgA will not only increase our knowledge on the recognition events in the plant secretory pathway but also have practical implications for the efficient production of active SIgA.

MATERIALS AND METHODS

In Vivo Labeling of Protoplasts and Analysis of Igs

The generation of transgenic tobacco (*Nicotiana tabacum*) plants expressing the monoclonal Guy's 13 IgG and the derivative, hybrid SIgA/G antibody has been described (Ma et al., 1994, 1995). Protoplasts were purified from leaves of transgenic plants expressing Guy's 13 IgG, IgA/G, dIgA/G, or SIgA/G as described (Pedrazzini et al., 1994). Pulse-chase labeling of protoplasts using a mixture of [^{35}S]Met and [^{35}S]Cys (Pro-Mix, Amersham Pharmacia Biotech, Little Chalfont, UK), cell homogenization and immunoprecipitation were performed as described previously (Pedrazzini et al., 1997). Treatment with brefeldin A

was performed by pre-incubating protoplasts for 45 min in the presence of 10 μg mL^{-1} brefeldin A (Boehringer Mannheim, Mannheim, Germany; stock solution 2 mg mL^{-1} in ethanol; a corresponding amount of ethanol was added to control protoplasts) and maintaining the same concentration of the drug for the entire pulse-chase labeling. Immunoprecipitated polypeptides were resolved onto 15% reducing or 6% (w/v) non-reducing SDS-PAGE. Gels were treated with 2,5-diphenyloxazole dissolved in dimethyl sulfoxide and radioactive proteins visualized by fluorography. Vacuolar purification was performed as described (Dombrowski et al., 1994). The recovery of vacuoles was around 30% based on α-mannosidase activity; this vacuolar fraction contained much less than 1% of the total cellular amount of the ER resident chaperone BiP, strongly suggesting very low contamination by other compartments of the secretory pathway (not shown).

For the isolation of microsomes, protoplasts were pulse-labeled for 1 h and precipitated by adding 3 volumes of W5 medium (154 mM NaCl, 5 mM KCl, 125 mM $CaCl_2 2H_2O$, and 5 mM Glc). Cells were resuspended in Suc buffer (100 mM Tris [tris(hydroxymethyl)aminomethane]-HCl, pH 7.5, 10 mM KCl, 1 mM EDTA, and 12% [w/w] Suc) and lysed on ice by sonication. The homogenate was centrifuged for 5 min at 1,000*g* to remove debris and intact cells. The supernatant was loaded on top of a 17% (w/w) Suc pad and centrifuged for 30 min at 150,000*g* in a TLA100 rotor (Beckman Instruments, Fullerton, CA), 4°C. The supernatant (containing cytosolic and vacuolar proteins) was removed and the microsomal pellet was resuspended in Suc buffer, in Suc buffer containing 1 M NaCl, or in 0.1 M Na_2CO_3. After 30 min of incubation on ice, the microsome suspensions were reloaded on top of a 17% (w/w) Suc pad and centrifuged for 30 min at 150,000*g*. Supernatants and pellets were homogenated in (final) 100 mM Tris-Cl, 100 mM NaCl, 1 mM EDTA, and 1% (v/v) Triton X-100, pH 7.5, supplemented with protease inhibitor cocktail (Complete, Boehringer Mannheim). Igs were immunoprecipitated with anti-IgG antiserum and analyzed by 6% (w/v) non-reducing SDS-PAGE and fluorography.

Immunoelectron Microscopy

Leaf specimens were taken from transgenic tobacco plants expressing SIgA/G grown in standard greenhouse conditions for 2 months. For ultrastructural analysis, leaf strips were first fixed with 4% (w/v) paraformaldehyde, 1% (w/v) glutaraldehyde in 100 mM phosphate buffer, pH 7.4, containing 5% (w/v) Suc. After rinsing in the same buffer the leaf strips were fixed again in 1% (w/v) osmium tetraoxide in buffer. Leaf strips were washed and dehydrated through an ethanol series, and embedded in Spurr's resin. Ultrathin sections of 75 nm were stained with 2% (w/v) uranyl acetate and 0.1% (w/v) lead citrate. For ultrastructure immunocytochemistry, leaf strips were fixed with 4% (w/v) paraformaldehyde and 1% (w/v) glutaldehyde. After rinsing and dehydration, the leaf specimens were embedded in LR White resin as described (Robinson et al., 1994) except all the infiltration steps were done at room temperature and the resin was polymerized at 52°C. Ultrathin sections (silver and gold in color) were collected onto formvar-coated nickel grids, dried in air, and used in double-antibody labeling. Grids were wetted in double-distilled water and etched with saturated sodium periodate for 30 min to unmask excessive fixation. After rinsing in double distilled water, the grids were blocked with 1.5% (w/v) chicken egg albumin in phosphate buffered saline (PBS: 150 mM NaCl and 10 mM potassium phosphate, pH 7.4) for 30 min. After blocking, grids were incubated with sheep anti-rabbit secretory antibody diluted in chicken egg albumin containing blocking buffer for 1 h. This first primary antibody was rinsed away by floating the grids in PBS for 15 min. After blocking in the block buffer for 15 min, the grids were incubated with a second primary antibody, affinity-purified rabbit anti-mouse Ig (IgG + IgA + IgM, heavy and light chain; Zymed, Carlton Court, CA) diluted in the blocking buffer for 1 h. The grids were floated in PBS for 15 min to wash away the second primary antibody and blocked with 5% (v/v) normal donkey serum diluted in PBS for 30 min. Grids were then incubated with gold (12 nm)-conjugated donkey anti-sheep Ig antibody (Jackson ImmunoResearch Lab, West Grove, PA) and gold (6 nm)-conjugated donkey anti-rabbit Ig antibody diluted in PBS containing 5% (v/v) normal donkey serum for 1 h. Grids were rinsed in PBS, then in double-distilled water and air-dried. Sections were post-stained in 2% (w/v) uranyl acetate. Electron micrographs were taken with an electron microscope (H-600, Hitachi, Tokyo).

Immunofluorescence Microscopy

After purification, protoplasts from either IgG or SIgA/G-expressing plants were resuspended in MaCa buffer (0.5 M mannitol, 20 mM $CaCl_2$, and 0.1% [w/v] MES, pH 5.7) at a concentration of 5 × 10^5 cells mL^{-1}. Three hundred microliters of cell suspension was spread onto poly-Lys-coated slides (Sigma, St. Louis) and cells were allowed to adhere for 30 min at room temperature. Cells were fixed for 30 min at room temperature in MaCa buffer containing 4% (w/v) paraformaldehyde. Cells were then permeabilized by washing three times with TSW buffer (10 mM Tris-HCl, pH 7.4, 0.9% [w/v] NaCl, 0.25% [w/v] gelatin, 0.02% [w/v] SDS, and 0.1% [v/v] Triton X-100) for 10 min at room temperature. Incubation with rabbit anti-IgG antiserum (Sigma, dilution 1:300) or anti-BiP antiserum (Pedrazzini et al., 1997; dilution 1:1,000) was in the same buffer for 1 h at room temperature. After three washes in TSW, cells were incubated for 1 h at room temperature with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody (Sigma) at a dilution of 1:200. After three final washes in TSW, cells were mounted in Mowiol (Calbiochem, San Diego) supplemented with 2.5% (w/v) DABCO (Sigma) as an antifade agent. Cells were visualized with a Bio-Rad MRC1024 confocal laser scanning microscope equipped with a 40X oil immersion objective, at 494 nm excitation and 520 nm emission. Thickness of the optical sections was 2 μm. Laser intensity and gain controls were used as indicated in the legend to Figure 6.

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LITERATURE CITED

Barrieu F, Chrispeels MJ (1999) Delivery of a secreted soluble protein to the vacuole via a membrane anchor. Plant Physiol 120: 961–968

Bednarek SY, Wilkins TA, Dombrowski JA, Raikhel NV (1990) A carboxyl-terminal propeptide is necessary for proper sorting of barley lectin to vacuoles of tobacco. Plant Cell 2: 1145–1155

Boevink P, Oparka K, Santa Cruz S, Martin B, Batteridge A, Hawes C (1998) Stacks on tracks: the plant Golgi apparatus traffics on an actin/ER network. Plant J 15: 441–447

Brodsky JL, McCracken AA (1999) ER protein quality control and proteasome-mediated protein degradation. Cell Dev Biol 10: 507–513

Chillaron J, Haas IG (2000) Dissociation from BiP and retrotranslocation of unassembled immunoglobulin light chains are tightly coupled to proteasome activity. Mol Biol Cell 11: 217–226

Coleman CE, Herman EM, Takasaki T, Larkins BA (1996) The maize γ-zein sequesters α-zein and stabilizes its accumulation in protein bodies of transgenic tobacco endosperm. Plant Cell 8: 2335–2345

Conrad U, Fiedler U (1998) Compartment-specific accumulation of recombinant immunoglobulins in plant cells: an essential tool for antibody production and immunomodulation of physiological functions and pathogen activity. Plant Mol Biol 38: 101–109

Crofts AJ, Leborgne-Castel N, Hillmer S, Robinson DG, Phillipson B, Carlsson LE, Ashford DA, Denecke J (1999) Saturation of the endoplasmic reticulum retention machinery reveals anterograde bulk flow. Plant Cell 11: 2233–2247

Denecke J, Botterman J, Deblaere R (1990) Protein secretion in plant cells can occur via a default pathway. Plant Cell 2: 51–59

Dombrowski JE, Gomez L, Chrispeels MJ, Raikhel NV (1994) Targeting of proteins to the vacuole. *In* SB Gelvin, RA Schilperoort, eds, Plant Molecular Biology Manual, Ed 2. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 1–29

Dombrowski JE, Schroeder MR, Bednarek SY, Raikhel NV (1993) Determination of the functional elements within the vacuolar targeting signal of barley lectin. Plant Cell 5: 587–596

Frigerio L, de Virgilio M, Prada A, Faoro F, Vitale A (1998) Sorting of phaseolin to the vacuole is saturable and requires a short C-terminal peptide. Plant Cell 10: 1031–1042

Fujiki Y, Hubbard AL, Fowler S, Lazarow PB (1982) Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. J Cell Biol 93: 97–102

Gomez L, Chrispeels MJ (1993) Tonoplast and soluble vacuolar proteins are targeted by different mechanisms. Plant Cell 5: 1113–1124

Guenzi S, Fra AM, Sparvoli A, Bet P, Rocco M, Sitia R (1994) The efficiency of cysteine-mediated intracellular retention determines the differential fate of secretory IgA and IgM in B and plasma cells. Eur J Immunol 24: 2477–2482

Hiatt A, Cafferkey R, Bowdish K (1989) Production of antibodies in transgenic plants. Nature 342: 76–78

Hinz G, Menze A, Hohl I, Vaux D (1997) Isolation of prolegumin from developing pea seeds: its binding to endomembranes and assembly into prolegumin hexamers in the protein storage vacuole. J Exp Bot 48: 139–149

Hong E, Davidson AR, Kaiser CA (1996) A pathway for targeting soluble misfolded proteins to the yeast vacuole. J Cell Biol 135: 623–633

Hunt DC, Chrispeels MJ (1991) The signal peptide of a vacuolar protein is necessary and sufficient for the efficient secretion of a cytosolic protein. Plant Physiol 96: 18–25

Ma JK-C, Hein MB (1995) Immunotherapeutic potential of antibodies produced in plants. Trends Biotechnol 13: 522–527

Ma JK-C, Hiatt A, Hein M, Vine ND, Wang F, Stabila P, von Dolleweerd C, Mostov K, Lehner T (1995) Generation and assembly of secretory antibodies in plants. Science 268: 716–719

Ma JK-C, Hikmat BY, Wycoff K, Vine ND, Chargelegue D, Yu L, Hein MB, Lehner T (1998) Characterization of a recombinant plant monoclonal secretory antibody and preventive immunotherapy in humans. Nat Med 4: 601–606

Ma JK-C, Lehner T, Stabila P, Fux CI, Hiatt A (1994) Assembly of monoclonal antibodies with IgG1 and IgA heavy chain domains in transgenic tobacco plants. Eur J Immunol 24: 131–138

Mancini R, Fagioli C, Fra AM, Maggioni C, Sitia R (2000) Degradation of unassembled soluble Ig subunits by cytosolic proteasomes: evidence that retrotranslocation and degradation are coupled events. FASEB J 14: 769–778

Marty F (1999) Plant vacuoles. Plant Cell 11: 587–599

Matsuoka K, Bassham DC, Raikhel NV, Nakamura K (1995) Different sensitivity to wortmannin of two vacuolar sorting signals indicates the presence of distinct sorting machineries in tobacco cells. J Cell Biol 130: 1307–1318

Matsuoka K, Nakamura K (1991) Propeptide of a precursor to a plant vacuolar protein required for vacuolar targeting. Proc Natl Acad Sci USA 88: 834–838

Mestecky J, McGhee JR (1987) Immunoglobulin A (IgA): molecular and cellular interactions involved in IgA biosynthesis and immune response. Adv Immunol 40: 153–245

Neuhaus J-M, Rogers JC (1998) Sorting of proteins to vacuoles in plant cells. Plant Mol Biol 38: 127–144

Pedrazzini E, Giovinazzo G, Bielli A, de Virgilio M, Frigerio L, Pesca M, Faoro F, Bollini R, Ceriotti A,

Vitale A (1997) Protein quality control along the route to the plant vacuole. Plant Cell 9: 1869–1880

Pedrazzini E, Giovinazzo G, Bollini R, Ceriotti A, Vitale A (1994) Binding of BiP to an assembly-defective protein in plant cells. Plant J 5: 103–110

Pedrazzini E, Villa A, Borgese N (1996) A mutant cytochrome b_5 with a lengthened membrane anchor escapes from the endoplasmic reticulum and reaches the plasma membrane. Proc Natl Acad Sci USA 93: 4207–4212

Pueyo JJ, Chrispeels MJ, Herman EM (1995) Degradation of transport-competent destabilized phaseolin with a signal for retention in the endoplasmic reticulum occurs in the vacuole. Planta 196: 586–596

Rayon C, Lerouge P, Faye L (1998) The protein *N*-glycosylation in plants. J Exp Bot 49: 1463–1472

Robinson DL, Kahn ML, Vance CP (1994) Cellular localization of nodule-enhanced aspartate aminotransferase in *Medicago sativa* L. Planta 192: 202–210

Saalbach G, Jung R, Kunze G, Saalbach I, Adler K, Müntz K (1991) Different legumin protein domains act as vacuolar targeting signals. Plant Cell 3: 695–708

Sanderfoot AA, Raikhel NV (1999) The specificity of vesicle trafficking: coat proteins and SNAREs. Plant Cell 11: 629–641

Schroeder MR, Borkhsenious ON, Matsuoka K, Nakamura K, Raikhel NV (1993) Colocalization of barley lectin and sporamin in vacuoles of transgenic tobacco plants. Plant Physiol 101: 451–458

Sitia R, Neuberger M, Alberini C, Bet P, Fra A, Valetti C, Williams G, Milstein C (1990) Developmental regulation of IgM secretion: the role of the carboxy-terminal cysteine. Cell 60: 781–790

Tabe LM, Wardley-Richardson T, Ceriotti A, Aryan A, McNabb W, Moore A, Higgins TJV (1995) A biotechnological approach to improving the nutritive value of alfalfa. J Anim Sci 73: 2752–2759

Underdown BJ, Dorrington KJ (1974) Studies on the structural and conformational basis for the relative resistance of serum and secretory immunoglobulin A to proteolysis. J Immunol 112: 949–959

Vitale A, Denecke J (1999) The endoplasmic reticulum: gateway of the secretory pathway. Plant Cell 11: 615–628

Vitale A, Raikhel NV (1999) What do proteins need to reach different vacuoles? Trends Plant Sci 4: 149–155

Wandelt CI, Khan MRI, Craig S, Schroeder HE, Spencer D, Higgins TJV (1992) Vicilin with carboxy-terminal KDEL is retained in the endoplasmic reticulum and accumulates to high levels in the leaves of transgenic plants. Plant J 2: 181–192

Transgenic Research 9: 187–194, 2000.

A murine monoclonal antibody produced in transgenic plants with plant-specific glycans is not immunogenic in mice

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Abstract

Previous studies have shown that the production of recombinant antibodies in plants is highly efficient and presents numerous therapeutic applications. It is, however, known that plant glycoproteins display different glycosylation patterns to those exhibited by mammalian glycoproteins. Thus, it is important to know if these plant recombinant antibodies could induce undesirable immune responses in mammals; and to date no report has documented the potential immunogenicity of parenterally administered plant recombinant antibodies in animals. In order to answer this question, mice were immunised subcutaneously with a recombinant mouse monoclonal antibody produced in tobacco plants, together with alum as adjuvant. Two control groups were immunised in the same way with either the original murine monoclonal antibody or horseradish peroxidase (a plant glycoprotein). Analyses by direct immunoassay, competition immunoassay and real-time surface plasmon resonance, showed undetectable levels of antibody directed against both the protein and the glycan part of the plant recombinant antibody. These results have a direct relevance for the application of plant recombinant proteins as therapeutic agents and vaccines in humans.

Introduction

The expression of recombinant antibodies in plants was first described a decade ago (Hiatt et al., 1989). There are several advantages in the use of plants for the production of recombinant proteins such as antibodies, for example, plant cells can express, fold, assemble and glycosylate full-length antibodies extremely efficiently (Ma & Hein, 1996; Ma et al., 1994; Hiatt & Ma, 1993), yielding functional recombinant antibody levels of 1–8% of total soluble leaf protein. For applications such as passive immunotherapy (Ma et al., 1998) where large quantities of antibody would be required, the potential for scaling up production to agricultural levels is also particularly attractive.

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For optimal expression in plants, the recombinant immunoglobulin chains are targeted to the plant endoplasmic reticulum and the secretory pathway where folding and assembly takes place as well as glycosylation. In common with other heterologous expression systems, plant glycosylation differs from that found in the native cell. Although high mannose type glycans are identical in plants and animals, there are differences between the complex glycans (Driouich et al., 1993). For example, plants (like insects and yeast) do not incorporate sialic acid, in addition, the complex glycans are characterised by a β1 → 2 xylose residue linked to the β-mannose and α1 → 3 fucose residue linked to the proximal glucosamine (Lerouge et al., 1998).

Recently, the *N*-glycan composition of a murine monoclonal IgG1 (Guy's 13) was determined and compared with the *N*-glycan structures associated with the same monoclonal antibody expressed in

plants (Cabanes-Macheteau et al., 1999). The same *N*-glycosylation sites were utilised in both expression systems, however, there was a greater diversity of glycoforms in the plant-derived antibody than the mouse-derived version. Compared with four different complex type oligosaccharide structures on the mouse Mab, there were eight different oligosaccharides on the plant Mab, with structurally related oligosaccharides ranging from high-mannose type (40%) to modified glycans (60%). Some similarities were found between the complex type *N*-glycans from both expression systems, such as the common Man GlcNAc2 cores substituted by terminal GlcNAc residues. However, the plant glycans also contained the characteristic terminal β1 → 2 xylose and α1 → 3 fucose residues that have been identified previously in plant glycoproteins.

It has been suggested that plant specific complex glycan structures may be highly immunogenic, which could be a limitation to the use of plants as a recombinant protein expression system for pharmaceutical production for humans (Faye et al., 1993; Driouich et al., 1993). Indeed, immunisation of rabbits with β-fructosidase, horseradish peroxidase, peanut peroxidase, or plant lectins (e.g. *Wistaria floribunda)* induced antibody responses that were specific against the two glycan motifs – α1 → 3 fucose and β1 → 2 xylose (Faye et al., 1993; Wilson et al., 1998; Wan & van Huystee, 1994; Ramirez-Soto & Poretz, 1991). Furthermore, it has been possible to affinity-purify antibodies for each specificity (Faye et al., 1993). So far, however, these studies have been limited to studying the immunogenicity of plant glycoproteins, and it has not been established that plant glycans remain immunogenic when presented in the context of mammalian or self-proteins. For example, carbohydrates in themselves are generally poor immunogens due to the absence of T-cell epitopes (Seppala & Makela, 1997).

In a human clinical trial in which a modified version of the mouse Guy's 13 Mab expressed in plants was administered on six occasions, no anti-plant antibody response was detected (Ma et al., 1998). In this study, however, the antibody was delivered topically in the oral cavity and the results would not necessarily reflect the response if the antibody was delivered systemically. If recombinant plant antibodies are to be used in systemic applications, it would be important to determine the potential immunogenicity of plant glycans. The purpose of this study was to investigate the immunogenicity of a plant recombinant monoclonal murine IgG antibody in mice when administered parenterally. To increase the likelihood of generating an immune response, the immunisations were carried out with alum as an adjuvant. Groups of animals were immunised either with the mouse derived Guy's 13 IgG1κ monoclonal antibody, the same Guy's 13 antibody produced in transgenic plants and bearing plant specific glycans, a standard plant glycoprotein (horseradish peroxidase) or phosphate buffered saline; and specific antibody responses in each animal were determined by ELISAs and biosensor.

Materials and methods

Monoclonal antibodies and antisera

Mouse monoclonal antibody (Mab) Guy's 13 (IgG1κ) is specific for *Streptococcus mutans* surface antigen I/II (Lehner et al., 1985). The murine derived antibody was purified from ascitic fluid by protein A affinity chromatography (Biogenesis, Poole, UK). Gene constructs encoding the light (κ) and heavy (γ_1) chains for Mab Guy's 13, their expression and co-assembly to form functional antibody in tobacco plants (plant rIgG1κ) have been previously described (Ma et al., 1994). Rabbit anti-plant lectin (*Canavalia ensiformis* and *Ricinus communis*) immune-sera were obtained from Sigma (Poole, UK). Rabbit immune-sera specific for horseradish peroxidase (HRP) were kindly provided by Dr Loïc Faye (Institute for Peptide Research, CNRS, University of Rouen, France).

Purification of plant recombinant IgG1κ

For purification of plant rIgG1κ, tobacco plant leaves were homogenised on ice with extraction buffer (37.5 mM Tris–HCl pH 7.5, 50 mM NaCl, 15 mM EDTA, 75 mM sodium citrate, 3% polyvinylpolypyrrolidone, 0.2% sodium thiosulfate and 10 μg/ml leupeptin). The suspension was filtered through a cheesecloth and its pH reduced to 5.1 by addition of 8 M acetate-NaOH buffer pH 3.8. The solution was centrifuged at 17,000 g for 20 min at 4°C. The pH of the solution was brought back to 7.0 by addition of 1.5 M Tris–HCl pH 9.5, and ammonium sulphate was added to 16% saturation. After centrifugation at 17,000 g for 20 min at 4°C, the precipitate was discarded, ammonium sulphate was added to the supernatant to 40% saturation. After at least 2 h at 4°C, the solution was centrifuged again, the pellet was resuspended in PBS to 1/5 of original volume, and the final

solution was then centrifuged at 25,000 g for 30 min at 4°C. The protein solution was concentrated by ultrafiltration through a 30 kDa molecular weight cut-off membrane (Amicon, UK) and dialysed overnight at 4°C against PBS. The concentrated extract was then filtered through a 0.45 μm filter and loaded onto a HiTrap Protein G column (Pharmacia, Uppsala, Sweden). The rIgG1κ was eluted with 0.1 M glycine-HCl pH 2.7 and neutralised with 1.5 M Tris–HCl pH 9.5. The preparation was then loaded onto a sheep anti-mouse IgG1 sepharose column (Sigma), eluted with glycine-HCl 0.1 M pH 2.5, neutralised with 1.5 M Tris–HCl pH 9.5, dialysed overnight against PBS and passed through a 0.22 μm filter. Finally, the protein concentration was estimated by measuring the absorbance at 280 nm (A_{280} = 1.0 for IgG concentration of 1.4 mg/ml) and the purity of the preparation assessed by SDS-PAGE.

SDS-PAGE

For SDS-PAGE analyses, the purified plant rIgG1κ samples were boiled for 3 min in 75 mM Tris–HCl (pH 6.8) containing 2% SDS and 5% 2-mercaptoethanol, separated on a 10% acrylamide gel and proteins were stained with Coomassie blue.

Animals and immunisation schedules

Inbred 6–7 week-old female BALB/c (H-2^d) mice were purchased from Charles Rivers, UK. Previous studies, had determined that a subcutaneous (s.c.) injection of 25–30 μg of antigen in alum or IFA (incomplete Freund's adjuvant) followed by two boosts, were optimal to obtain >3 Log_{10} of high affinity IgG titre, 4–5 weeks after the last injection (Chargelegue et al., 1998; Obeid et al., 1996; Hsu et al., 1999). Thus in this investigation, a similar schedule was adopted: four groups of six mice were immunised s.c. at the base of the tail, with either PBS, 30 μg of mouse IgG1κ, plant rIgG1κ, or affinity purified HRP (Sigma, UK) in 50% alum suspension (Alu-Gel-S, Serva, Boehringer Ingelheim, Germany). The mice were boosted twice at 3 and 9 weeks, with the same dose of immunogen in alum and the final bleeds were collected 4–5 weeks after the last boost. Pre-immune sera were used as negative control samples in all the analyses.

Biosensor analyses

Biosensor analyses were done using the BIAcore X system (BIAcore AB, Sweden). Approximately 1000 resonance units (RU) of IgG1κ, plant rIgG1κ or HRP were immobilised on a CM5 sensorship (BIAcore) using a NHS/EDC (*N*-hydroxysuccinimide/*N*-ethyl-*N'*-(dimethylaminopropyl) carbodiimide) chemistry (BIAcore). The binding kinetics of mouse and rabbit immune-sera were measured at a dilution of 1/5 in HBS-tween 0.005% (BIAcore). After each binding measurement, the chip was regenerated with 0.1 M HCl-glycine pH 2.0.

Enzyme Immunoassays

Maxisorp 96-well microplates (Nunc, Roskilde, Denmark) were coated with 50 μl of IgG1κ, plant rIgG1κ or HRP at 5 μg/ml in PBS pH 7.4, overnight at 4°C, washed with water and blocked with 200 μl/well of PBS-BSA 2.5% for 2 h at 37°C. After washing, the plates were incubated with two-fold dilution (from 1/20 onwards) of mouse sera or rabbit immune sera in PBS-Tween 20 (0.1%) BSA (2.5%) (diluting buffer) for 2 h at 37°C and washed. Plates were incubated with appropriate antisera: sheep anti-mouse IgG (The Binding Site, Birmingham, UK), sheep anti-mouse IgG2a + IgG2b + IgG3 (The Binding Site), goat anti-mouse Ig lambda chain (Caltag, Burlingame, CA) or sheep anti-rabbit IgG (Boehringer Mannheim, Lewes, UK), in diluting buffer, for 1 h at 37°C. These secondary antibodies were either conjugated with HRP or alkaline phosphatase (AP). For HRP-conjugates, enzyme activity was detected by addition of 100 μl of substrate solution (0.01% of 3,3',5,5'-tetramethylbenzidine and 0.004% hydrogen peroxide in citrate-phosphate buffer, pH 5.0). After 10 min at room temperature the enzymatic reaction was stopped with 50 μl of 2 M H_2SO_4 and the OD was determined at 450 nm. For AP-conjugates, enzyme activity was detected by addition of 100 μl of substrate solution (0.1% *p*-nitrophenylphosphate in Tris–HCl Buffer pH 9.5 containing 5 mM $MgCl_2$). The reaction was stopped with 50 μl NaOH 10 M after at least 30 min incubation at room temperature and the plates were read at 405 nm.

For the competition ELISAs (Chargelegue et al., 1998; Steward and Chargelegue, 1997), 50 μl of serial dilution of mouse sera and 50 μl of anti-HRP or anti-plant lectins rabbit immune-sera (at a predetermined optimal working dilution to give a final OD of 0.8–1.0) were incubated overnight at 4°C with HRP or plant rIgG1κ coated plates. After washing, bound rabbit antibodies were reacted with anti-rabbit AP or HRP conjugates (Roche diagnostics, UK), 1 h at

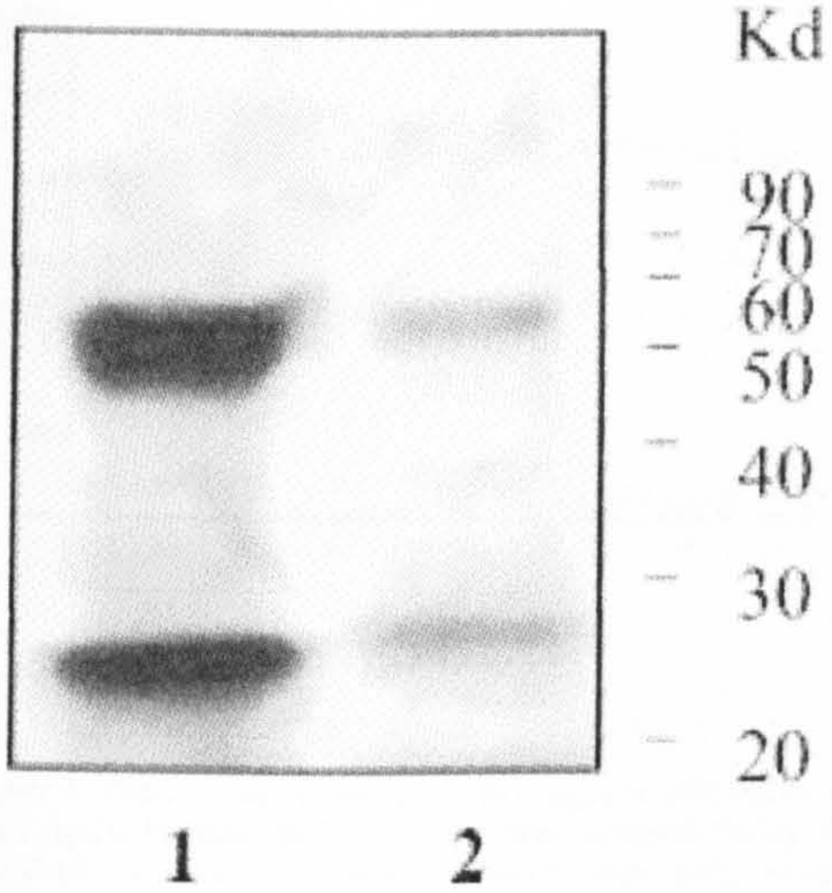

Figure 1. SDS-PAGE of the purified recombinant plant IgG1κ. Two loading amounts of protein are shown. Lane 1: 100 μg; Lane 2: 20 μg.

37°C. The plates were developed and read as described above. The inhibition titre was expressed as the $\log_{10}$ of the reciprocal highest dilution of mouse sera giving a 25% inhibition of the rabbit antiserum.

Results

Purification of plant rIgG1κ (Guy's 13)

Recombinant IgG1κ was purified from mature tobacco plants by ammonium sulphate precipitation and a succession of affinity chromatographies (protein G and anti-IgG1 columns). The yield of antibody was comprised between 1 and 2.5 mg/kg of fresh plant leaves. On SDS-PAGE analysis (Figure 1), the purified antibody consisted of two major bands (heavy and light chains). Four other bands of very low intensity were observed as well, which were degradation products of heavy or/and light chains as confirmed by western-blot analyses (data not shown).

Antibody responses to IgG1κ, Plant rIgG1κ and HRP in mice

To assess the potential immunogenicity of plant rIgG1κ, groups of six BALB/c mice were immunised with either plant rIgG1κ, IgG1κ or HRP (control plant

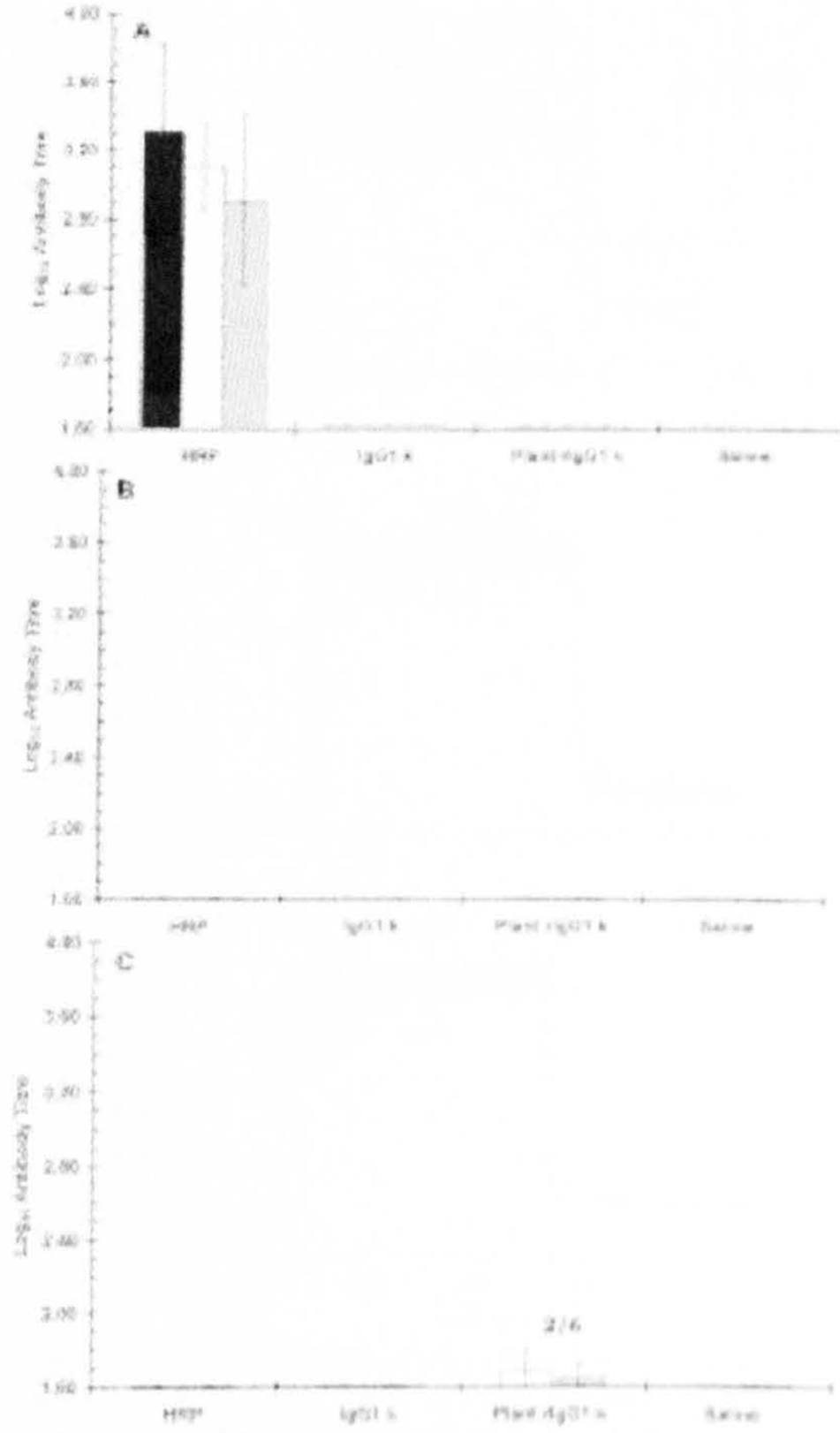

Figure 2. Antibody titres of sera from mice immunised with either HRP, IgG1κ, plant r IgG1κ or saline. Analysis of immune-reactivity to HRP (A), IgG1κ (B) and Plant rIgG1κ (C) by ELISA specific for: IgGs (black bar), $IgG_{2a,2b\&3}$ (clear bar) and Ig lambda chain (grey bar). Results are shown as mean ± standard deviation for six mice per group.

glycoprotein) at 30 μg/mouse in alum. As a negative control, a fourth group was sham immunised with PBS plus alum (saline group). A final bleed from each animal was obtained 4–5 weeks after the third immunisation. Antibody responses to HRP, plant rIgG1κ and IgG1κ, in each group were analysed by ELISA and supplemented by BIAcore studies.

Log_{10} antibody titres described in Figure 2 were obtained by serial doubling dilution of sera in an indirect enzyme-immunoassay. For the detection of antibody titres to HRP, secondary antibodies specific for all IgG subclasses (IgGs), for IgG(2a + 2b + 3), and for lambda chain could be used. All mice (6/6)

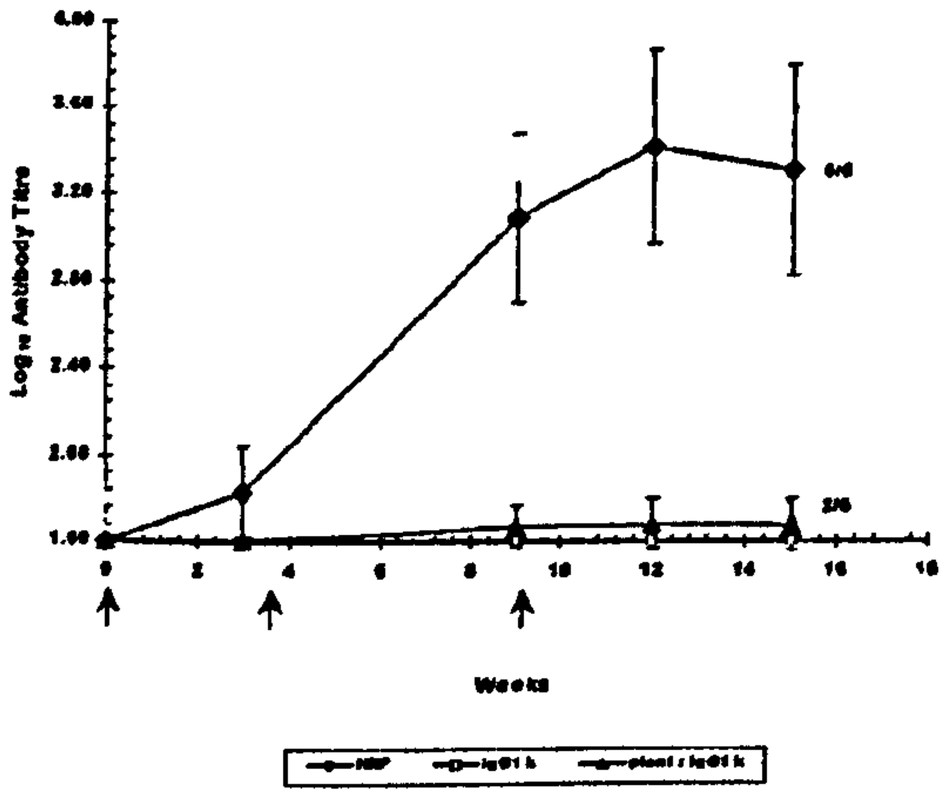

Figure 3. Time course of antibody titres (log_{10}) to HRP, IgG1κ and plant rIgG1κ in immunised mice. The arrows represent first, second and third immunisation at weeks 0, 3 and 9, respectively. Mean ± standard deviation are shown for six mice per group.

immunised with HRP had IgGs, IgG(2a+2b+3) and lambda log_{10} titres of 3.31 (±0.51), 3.11 (±0.26) and 2.91 (±0.49) respectively (Figure 2A). All the other groups had undetectable antibody titres to HRP (detection limit: log_{10} titre <1.60).

For the detection of antibody responses to IgG1κ and to plant rIgG1κ, only secondary antibodies specific for IgG(2a+2b+3) and lambda light chain could be used. There were no detectable antibody responses to IgG1κ in any of the immunised groups of animals (Figure 2B). Similar patterns were observed for reactivity to plant rIgG1κ (Figure 2C), but two of the six animals that were immunised with plant rIgG1κ had very weak antibody titres that were just above the limit of detection of the assay (IgG(2a+2b+3): 1.82±0.25; lambda: 1.70±0.15).

The time course of the antibody responses to HRP, IgG1κ and plant rIgG1κ was also assessed over 15 weeks (Figure 3). This analysis showed that the antibody titre to HRP rose from weeks 0 to 9, and reached a plateau between weeks 9 and 15. Responses in the plant rIgG1κ group were seen after the second immunisation in only two out of six animals. Antibody levels were very low throughout and were only just above the limits of detection for this assay.

The second part of the analysis was obtained by biosensor studies. This method allows measurement of real-time interaction between analyte and ligand without the use of secondary conjugates. Therefore, it should be possible to detect a total Ig (immunoglobulin) response against mouse IgG1κ or plant

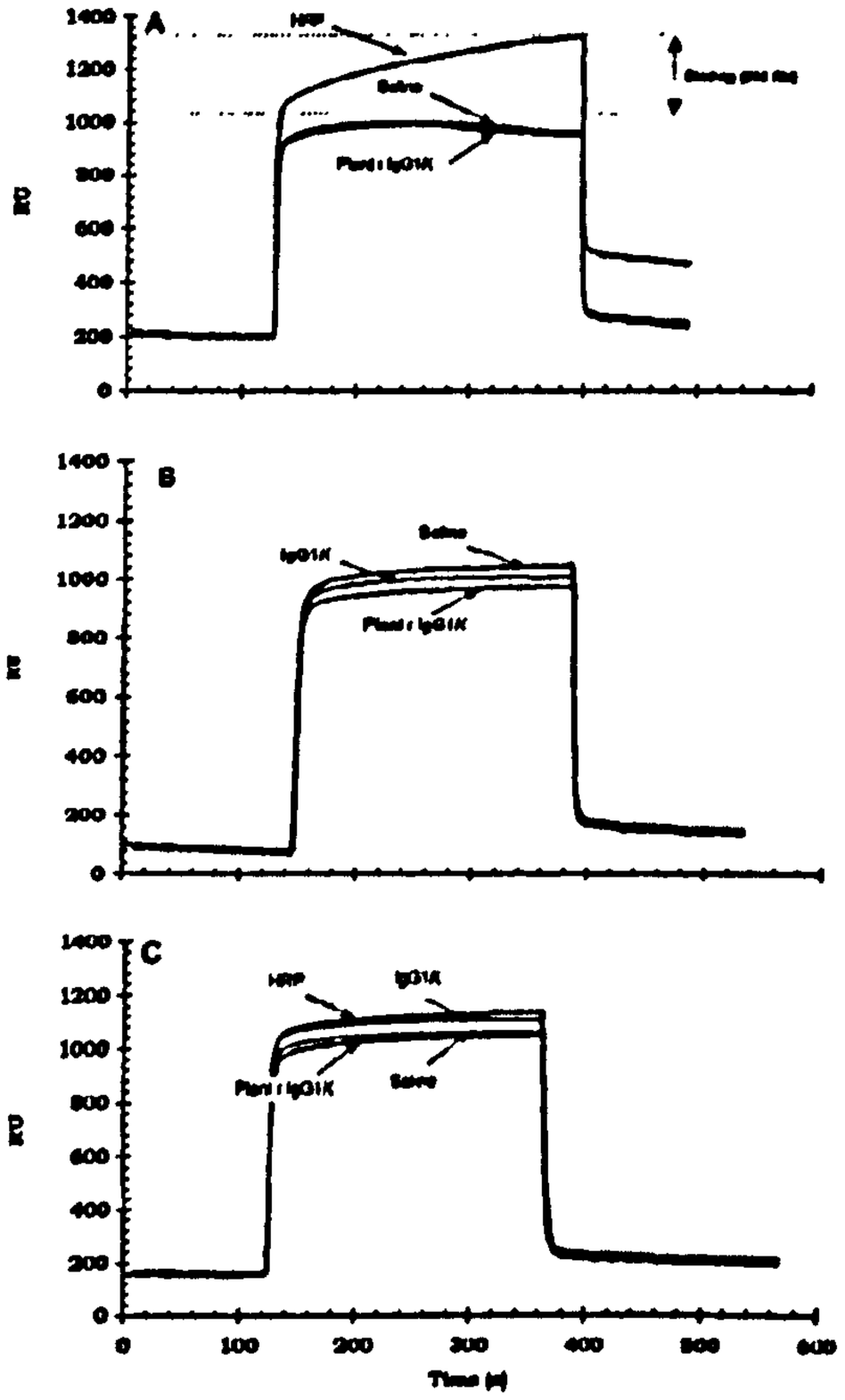

Figure 4. Antibody reactivities of sera from mice immunised with either HRP, IgG1κ, Plant rIgG1κ or saline by surface plasmon resonance. Sensorgrams for reactivity to HRP (A), IgG1κ (B) and Plant rIgG1κ (C) are shown.

rIgG1κ which could not be done by ELISA. Each curve in Figures 4A, 4B and 4C shows binding of pooled sera from each group to HRP, IgG1κ, and plant rIgG1κ, respectively. Experiments were also done on individual sera, and similar results were obtained (data not shown). As shown in Figure 4A, only sera from HRP immunised mice reacted significantly with HRP; 295 resonance units (RU) of binding for sera diluted at 1/5. No binding to HRP was observed for sera (at 1/5 dilution) from animals immunised with plant rIgG1κ or saline (Figure 4A). There was no significant reactivity to IgG1κ in any of the groups immunised with saline, IgG1κ or plant rIgG1κ (Figure 4B). Figure 4C represents the binding curves to plant rIgG1κ. No significant reactivity was observed in all groups immunised with HRP, plant rIgG1κ, IgG1κ or with saline.

Table 1. HRP and plant lectins rabbit immune-sera reactivity to IgG1κ, plant rIgG1κ and HRP: competition with sera from mice immunised with HRP and plant rIgG1κ

Rabbit immune-sera	Reactivity to IgG1κ IgG Log_{10} titre	Reactivity to plant rIgG1κ IgG Log_{10} titre	Competition with sera from mice immunised with plant rIgG1κ	Reactivity to HRP IgG Log_{10} titre	Competition with sera from mice immunised with HRP
Rabbit anti-HRP	<1.60	3.71	< 0.70[b]	5.63	2.10 ± (0.23)[a]
Rabbit anti-lectin 1	<1.60	3.61	<0.70[b]	4.91	<0.70[b]
Rabbit anti-lectin 2	<1.60	2.89	<0.70[b]	3.81	<0.70[b]

The working dilutions of rabbit anti-sera in the competition ELISA were optimised to give an OD comprised between 0.8 and 1.0.
[a]The value represents the Log_{10} of the reciprocal dilution of mouse sera dilution giving a 25% inhibition of rabbit anti-sera binding (average of six animals).
[b]When no inhibition of rabbit anti-sera was observed the value indicated is the Log_{10} of the reciprocal of the lowest mouse sera dilution tested (limit of detection of the assay).
Lectin 1: *Canavalia ensiformis*; Lectin 2: *Ricinus communis*.

Cross-reactivity to plant glycans (HRP and plant lectins)

Rabbit anti-HRP and plant lectin immune-sera have been shown to react strongly against α1 → 3 fucose and β1 → 2 xylose residues (Faye et al., 1993; Ramirez-Soto & Poretz, 1991). Thus, rabbit immune-sera raised against three different plant glycoproteins; HRP and two plant lectins (*Canavalia ensiformis* and *Ricinus communis*) were used in competition ELISAs to study the immune cross-reactivity to plant glycans of sera from mice immunised with plant rIgG1κ and HRP. It is important to note that the working dilutions of rabbit immune-sera in the competition-ELISA were optimised to obtain a sensitive assay; that is, working dilutions gave a final O.D. between 0.8 and 1.0 (Chargelegue et al., 1998; Steward & Chargelegue 1997). Rabbit anti-sera to HRP cross-reacted strongly with the plant rIgG1κ but not the mouse IgG1κ (Table 1), confirming the presence of anti-plant glycan antibodies. Furthermore, the two plant lectins anti-sera were highly cross-reactive against HRP (Table 1). This is consistent with the fact that plant lectins are *N*-glycosylated and display a range of plant specific *N*-linked oligosaccharide structures (Wilson et al., 1998; Marcus et al., 1984; Ashford et al., 1991). However, in the competition ELISA, there was no detectable inhibition of rabbit anti-HRP antiserum binding to plant rIgG1κ by the sera from the mice immunised with plant rIgG1κ (including the two that had weak reactivity in the ELISA) (Table 1). Sera from mice immunised with HRP did inhibit this rabbit anti-serum binding to HRP, but the same mouse sera did not show any detectable inhibiting activity against the rabbit anti-plant lectin sera binding to HRP (Table 1).

Discussion

The expression and production of recombinant antibodies in plant are now well established (Hiatt et al., 1989; Ma & Vine, 1999). Furthermore, our group has recently shown that it was possible to use these recombinant antibodies topically in humans without side effects (Ma et al., 1998). However, for other medical applications it is evident that antibodies will have to be delivered systemically. Although plant recombinant antibodies display glycan residues that are characteristic of plant glycoproteins, little is known about the potential immunogenicity of these mammalian proteins expressing plant glycans.

In the present study, we have investigated the immunogenicity of a mouse Mab Guy's 13 produced in tobacco (plant rIgG1κ) when administered parenterally. The same strain of mice was used in these immunisation studies (BALB/c) as was used for generating the original monoclonal antibody. Thus the study specifically addresses the immunogenicity of a self protein displaying foreign plant glycan structures. Four groups of six mice were immunised either with saline, IgG1κ, plant rIgG1κ or HRP. To optimise the likelihood of eliciting an immune response, immunisation was performed in the presence of adjuvant (alum), although adjuvants are unlikely to be used in normal clinical applications of systemically administered antibodies.

As expected, none of the animals immunised with mouse IgG1κ raised detectable antibody responses, by ELISA or biosensor analyses, to IgG1κ, plant rIgG1κ or HRP. HRP possesses both of the predominant immunogenic plant carbohydrate residues, α1 → 3 fucose and β1 → 2 xylose (Faye et al., 1993), thus it was used as a positive control for plant glycopro-

tein. The group of mice immunised with HRP did raise high IgG titres to HRP, however these antibodies did not cross-react with plant rIgG1κ. Since, the plant rIgG1κ also displays α1 → 3 fucose and β1 → 2 xylose (Driouich et al., 1993), it is unlikely that the mouse antibodies were specific for these plant glycans. Indeed, this was confirmed by the fact that the mouse antisera did not inhibit the binding of rabbit anti-plant lectin antisera to HRP glycan residues.

In the group immunised with plant rIgG1κ, two out of six mice had low reactivity (just above the detection limit) to the plant rIgG1κ preparation in ELISA (IgG2a, 2b & 3 and lambda responses). However, this reactivity was not detectable in the biosensor analyses, even though total Ig responses (the four IgG subclasses and IgM) could theoretically be monitored. The reason for this difference could be that the sensitivity of biosensors is not as great as ELISA methods when low sera dilutions are used, due to non-specific binding of serum components to the sensorchip surface (Kubitschko et al., 1997).

Furthermore, when the two positive sera were tested (at a dilution of 1/5) for their reactivity to plant glycans, neither was able to inhibit the binding of the anti-HRP rabbit immune-sera to plant rIgG1κ. Thus it seems unlikely that the two animals mounted a response to glycans present on plant rIgG1κ. Since these two sera did not react to the mouse IgG1κ either, it is possible that the results represent reactivity to a low amount of tobacco plant protein present at the end of the double-step affinity purification of plant rIgG1κ. Analyses by western-blotting using the sera from immunised animals were performed, however no antibody reactivity to plant contaminants in the plant rIgG1κ preparation nor to whole plant extract was detectable (data not shown). It is important to note that the level of reactivity in ELISA was only just above the detection limit of the assay (sera diluted at 1/40, gave an O.D of around 0.3 for only two animals out of six after overnight incubation), so it is unlikely that this level of reactivity will be detectable in western-blotting.

An unexpected result of this study was that the six mice immunised s.c. with HRP in alum, had undetectable anti-plant glycan reactivities. Indeed, previous publications had suggested that both plant glycan residues α1 → 3 fucose and β1 → 2 xylose, are highly immunogenic when plant glycoproteins were administered parentally with adjuvant (Faye et al., 1993; Wilson et al., 1998; Wan & van Huystee, 1994; Ramirez-Soto & Poretz, 1991). However, all these studies have employed rabbit as the animal model and used Freund's complete and/or incomplete adjuvant. In our study, alum was chosen as the adjuvant, because it is licensed for human use in vaccine trials (Gellin et al., 1997; Francis et al., 1998).

In conclusion, our study has demonstrated that plant glycans are poorly immunogenic in mice even when administered parenterally with adjuvant. This appears to be true whether the glycans are associated with either foreign (HRP) or self proteins (plant rIgG1κ). In the present work, a weak reactivity to the plant rIgG1κ preparation was observed in only two animals. Since no antibody response to the protein (no response to IgG1κ) and carbohydrate part (no competition with anti-HRP immune-sera) of the plant rIgG1κ were detected, this is almost certainly due to the presence of tobacco plant compounds in small quantity at the end of the purification. Therefore, it will be important for future clinical work to ensure that these contaminants are removed before immunisation (e.g. by a final ion-exchange or gel filtration HPLC chromatography). The present study was a pilot investigation in experimental mice, and in future it is likely that any recombinant plant proteins for human application will need to be tested for immunogenicity in humans as part of Phase I/II trials.

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References

Ashford DA, Dwek RA, Rademacher TW, Lis H and Sharon N (1991) The glycosylation of glycoprotein lectins. Intra- and inter-genus variation in *N*-linked oligosaccharide expression. *Carbohydrate Res* 213: 215–227.

Cabanes-Macheteau M, Fitchette-Laine AC, Loutelier-Bourhis C, Lange C, Vine ND, Ma JK-C, Lerouge P and Faye L (1999) *N*-glycosylation of a mouse IgG expressed in transgenic tobacco plants. *Glycobiology* 9: 365–372.

Chargelegue D, Obeid OE, Hsu SC, Shaw MD, Denbury AN, Taylor G and Steward MW (1998) A peptide mimic of a protective epitope of respiratory syncytial virus selected from a combinatorial library induces virus-neutralizing antibodies and reduces viral load *in vivo*. *J Virol* 72: 2040–2046.

Driouich A, Faye L and Staehelin LA (1993) The plant Golgi apparatus: A factory for complex polysaccharides and glycoproteins. *Trends Biochem Sci* 18: 210–214.

Faye L, Gomord V, Fitchette-Laine A and Chrispeels MJ (1993) Affinity purification of antibodies specific for Asn-linked glycans containing alpha 1 → 3 fucose or beta 1 → 2 xylose. *Anal Biochem* **209**: 104–108.

Francis DP, Gregory T, McElrath MJ, Belshe RB, Gorse GJ, Migasena S, Kitayaporn D, Pitisuttitham P, Matthews T, Schwartz DH and Berman PW (1998) Advancing AIDSVAX to phase 3. Safety, immunogenicity, and plans for phase 3. *AIDS Res Hum Retroviruses* **14** (Suppl 3): S325–S331.

Gellin BG, Greenberg RN, Hart RH, Bertino JS, Stein DH, Deloria MA and Clements-Mann ML (1997) Immunogenicity of two doses of yeast recombinant hepatitis B vaccine in healthy older adults. *J Infect Dis* **175**: 1494–1497.

Hiatt A, Cafferkey R and Bowdish K (1989) Production of antibodies in transgenic plants. *Nature* **342**: 76–78.

Hiatt A and Ma JK (1993) Characterization and applications of antibodies produced in plants. *Int Rev Immunol* **10**: 139–152.

Hsu SC, Chargelegue D and Steward MW (1999) Combination of B- and T-cell epitopes based vaccine induces protection against RSV infection in mice. Synergistic effect of immunization with a peptide cocktail inducing antibody, helper and cytotoxic T-cell responses on protection against RSV. *J Gen Virol* **80**: 1401–1405.

Kubitschko S, Spinke J, Bruckner T, Pohl S and Oranth N (1997) Sensitivity enhancement of optical immunosensors with nanoparticles. *Anal Biochem* **253**: 112–122.

Lehner T, Caldwell J and Smith R (1985) Local passive immunization by monoclonal antibodies against streptococcal antigen I/II in the prevention of dental caries. *Infect Immun* **50**: 796–799.

Lerouge P, Cabanes-Macheteau M, Rayon C, Fischette-Laine AC, Gomord V and Faye L (1998) *N*-glycoprotein biosynthesis in plants: recent developments and future trends. *Plant Mol Biol* **38**: 31–48.

Ma JK, Lehner T, Stabila P, Fux CI and Hiatt A (1994) Assembly of monoclonal antibodies with IgG1 and IgA heavy chain domains in transgenic tobacco plants. *Eur J Immunol* **24**: 131–138.

Ma JK, Hikmat BY, Wycoff K, Vine ND, Chargelegue D, Yu L, Hein MB and Lehner T (1998) Characterization of a recombinant plant monoclonal secretory antibody and preventive immunotherapy in humans. *Nat Med* **4**: 601–606.

Ma JK and Hein MB (1996) Antibody production and engineering in plants. *Ann NY Acad Sci* **792**: 72–81.

Ma JK and Vine ND (1999) Plant expression systems for the production of vaccines. *Curr Top Microbiol Immunol* **236**: 275–292.

Marcus SE, Burgess J, Maycox PR and Bowles DJ (1984) A study of maturation events in Jackbeans (*Canavalia ensiformis*). *Biochem J* **222**: 265–268.

Obeid OE, Stanley CM and Steward MW (1996) Immunological analysis of the protective responses to the chimeric synthetic peptide representing T- and B-cell epitopes from the fusion protein of measles virus. *Virus Res* **42**: 173–180.

Ramirez-Soto D and Poretz RD (1991) The (1 → 3)-linked alpha-L-fucosyl group of the *N*-glycans of the *Wistaria floribunda* lectins is recognized by a rabbit anti-serum. *Carbohydr Res* **213**: 27–36.

Seppala LJT and Makela O (1997) Haptens and Carriers. In: Herzenberg LA, Weir DM and Blackwell C (eds) *Handbook of Experimental Immunology*. (p. 43.1) Blackwell Scientific, Oxford, UK.

Steward MW and Chargelegue D (1997) Antibody affinity: Measurement and biological significance. In: Blackwell C, Herzenberg LA and Weir DM (eds), *Handbook of experimental immunology*. (p. 35.1) Blackwell Scientific, Oxford, UK.

Wan L and van Huystee RB (1994) Immunogenicity of the *N*-glycans of peanut peroxidase. *Phytochemistry* **37**: 933–940.

Wilson IB, Harthill JE, Mullin NP, Ashford DA and Altmann F (1998) Core alpha1,3-fucose is a key part of the epitope recognized by antibodies reacting against plant *N*-linked oligosaccharides and is present in a wide variety of plant extracts. *Glycobiology* **8**: 651–661.

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N-Glycosylation of a mouse IgG expressed in transgenic tobacco plants

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Since plants are emerging as an important system for the expression of recombinant glycoproteins, especially those intended for therapeutic purposes, it is important to scrutinize to what extent glycans harbored by mammalian glycoproteins produced in transgenic plants differ from their natural counterpart. We report here the first detailed analysis of the glycosylation of a functional mammalian glycoprotein expressed in a transgenic plant. The structures of the N-linked glycans attached to the heavy chains of the monoclonal antibody Guy's 13 produced in transgenic tobacco plants (plantibody Guy's 13) were identified and compared to those found in the corresponding IgG1 of murine origin. Both N-glycosylation sites located on the heavy chain of the plantibody Guy's 13 are N-glycosylated as in mouse. However, the number of Guy's 13 glycoforms is higher in the plant than in the mammalian expression system. Despite the high structural diversity of the plantibody N-glycans, glycosylation appears to be sufficient for the production of a soluble and biologically active IgG in the plant system. In addition to high-mannose-type N-glycans, 60% of the oligosaccharides N-linked to the plantibody have β(1, 2)-xylose and α(1, 3)-fucose residues linked to the core $Man_3GlcNAc_2$. These plant-specific oligosaccharide structures are not a limitation to the use of plantibody Guy's 13 for topical immunotherapy. However, their immunogenicity may raise concerns for systemic applications of plantibodies in human.

Key words: monoclonal antibody/N-glycosylation/transgenic plants

Introduction

Transgenic plants are an important system for the expression of therapeutic recombinant proteins (Moffat, 1995). Hiatt *et al.* (1989) demonstrated that the coexpression in tobacco of light and heavy immunoglobulin chains results in the production of functional antibodies. Since then, a number of groups have expressed antibodies in plants, either to modify plant performance or to exploit plants as bioreactors for large-scale production of full-length IgG (During *et al.*, 1990; Ma *et al.*, 1994) and secretory antibodies (Ma *et al.*, 1995).

It has been shown that some of the properties of immunoglobulins depend on their glycosylation. In general, there is one conserved N-glycosylation site per heavy chain of IgG in the C_H2 domain constitutive of the Fc region (Rademacher *et al.*, 1986). Glycosylation of the Fab region has also been described in about 30% of serum antibodies (Rademacher *et al.*, 1986). The N-glycosylation of the Fc contribute to the structural stability of the immunoglobulin. The two constitutive oligosaccharides of an IgG molecule stabilize the Fc by filling the interstitial region between the two C_H2 domains (Parekh *et al.*, 1985) and exert a subtle influence on protein tertiary and quaternary structures that is essential for activity. Aglycosylated IgG usually shows higher sensitivity to proteases and a loss of binding capacity to monocyte Fc receptors (for recent reviews, see Jefferis and Lund, 1997; Wright and Morrison, 1997). As a consequence, the N-glycosylation of recombinant antibodies is one of the key steps for the production of fully functional immunoglobulins by an expression system.

The N-glycosylation in higher organisms is conserved but differs slightly in detail. The processing of the N-linked glycans occurs along the secretory pathway as the glycoprotein moves from the endoplasmic reticulum through the Golgi apparatus to its final destination. Glycosidases and glycosyltransferases located in the Golgi apparatus successively modify the oligosaccharide precursor to high-mannose-type N-glycans and then into complex-type N-glycans. The complex-type N-glycans arise from the transfer in the Golgi apparatus of monosaccharide residues onto the core $Man_3GlcNAc_2$ under the action of several glycosyltransferases. Since some of these modifications are specific for the expression system, the structure of mature complex-type N-glycans associated with plant, insect, yeast, or mammalian glycoproteins will differ. Thus, no heterologous system will be able to reproduce mammalian glycans exactly. Plants, insects, and yeast do not introduce sialic acid on their glycoproteins and synthesize N-glycans having carbohydrate motifs that are not found in mammals. For instance, in plants, complex-type N-glycans are characterized by the presence of β(1,2)-xylose residue linked to the β-mannose and/or an α(1,3)-fucose residue, instead of an α(1,6)-fucose residue, linked to the proximal glucosamine (for a recent review, see Lerouge *et al.*, 1998). Since plants are gaining acceptance for the expression of recombinant therapeutic proteins, it is important to examine in detail to what extent glycans of mammalian glycoproteins produced in transgenic plants differ from original ones.

The monoclonal antibody (MAb) Guy's 13 is a mouse IgG1 class antibody, which recognizes a cell-surface protein of *Streptococcus mutans*, the bacteria which is the principal cause of dental caries in humans (Smith and Lehner, 1989). This MAb contains two potential N-glycosylation sites on its constitutive heavy chain. In addition to the highly conserved glycosylation site in the Fc region, MAb Guy's 13 has a second Asn-74-Ser-Ser N-glycosylation consensus sequence located in the Fab part of this IgG1 molecule. A full-length MAb Guy's 13 was expressed in tobacco (Ma *et al.*, 1994). This plant MAb or plantibody was found to be functional in terms of antigen recognition and binding. In this article, we report a detailed structural comparison

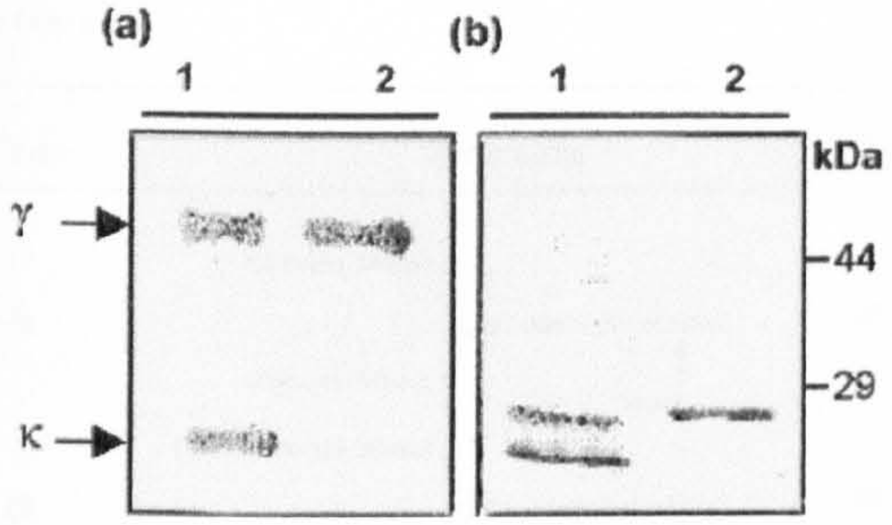


Fig. 1. SDS–PAGE and Western blot analysis of the mouse MAb Guy's 13 and Fab fragment. The purified mouse MAb Guy's 13 (**a**) and Fab fragment (**b**) were analyzed by SDS–PAGE in reducing conditions and detected by silver staining in the gel (lanes 1) or by affinodetection with RCA of the corresponding blots (lanes 2). γ, Guy's 13 heavy chain; κ, Guy's 13 light chain.

of the N-glycans of the MAb Guy's 13 expressed in mouse and in tobacco plants using immunochemical and physicochemical approaches.

Results

N-Glycosylation analysis of the mouse Guy's 13 antibody

The MAb Guy's 13 has two potential N-glycosylation sites located on the heavy chain: the conserved site in the Fc fragment and an additional site on Asn-74 located on the Fab fragment. The structure of the N-linked glycans and their distribution on the two potential N-glycosylation sites were first investigated by affinodetection on blots using *Ricinus communis* agglutinin (RCA). This lectin specifically binds to N-acetyllactosamine sequence usually found in mammalian complex-type N-glycans. Heavy and light chains constitutive of the mouse IgG and the Fab fragment were separated by SDS–PAGE in reducing conditions, and they were affinodetected on blots (Figure 1). In both the IgG and the Fab, the heavy chain was found to be detected by RCA on blots (Figure 1, lanes 2). As a consequence, in addition to the oligosaccharide attached to the conserved N-glycosylation site of the Fc, the site (Asn-74) located on the Fab is N-glycosylated by RCA-positive oligosaccharides.

The monosaccharide analysis of the mouse MAb was performed in two steps. The neutral and amino sugars were released by hydrolysis with 2 M TFA. The identification and quantification of monosaccharides were done by comparison to calibrated standards as previously reported (Hardy, 1989). Fuc, Gal, GlcNAc, and Man were detected in the molar ratio 0.65:1.5:3.8:3.0. Neuraminic acid released in mild acidic conditions (0.2 M TFA) was estimated to represent about 10% of the sugar content (Anumula and Taylor, 1991). The structures of N-linked glycans attached to the mouse MAb were determined by chromatographic analysis and nuclear magnetic resonance (NMR). The oligosaccharides were released from the MAb with PNGase F and analyzed by HPAE-PAD chromatography as reported in Rohrer *et al.* (1995). The mouse MAb N-linked glycans were found to be separated into neutral and sialylated oligosaccharides in a 9:1 ratio, confirming that sialylated N-linked glycans represent about 10% of the total oligosaccharides (Figure 2A). Total N-glycans isolated from the mouse MAb were coupled to 2-aminopyridine, and the resulting pyridylamino-

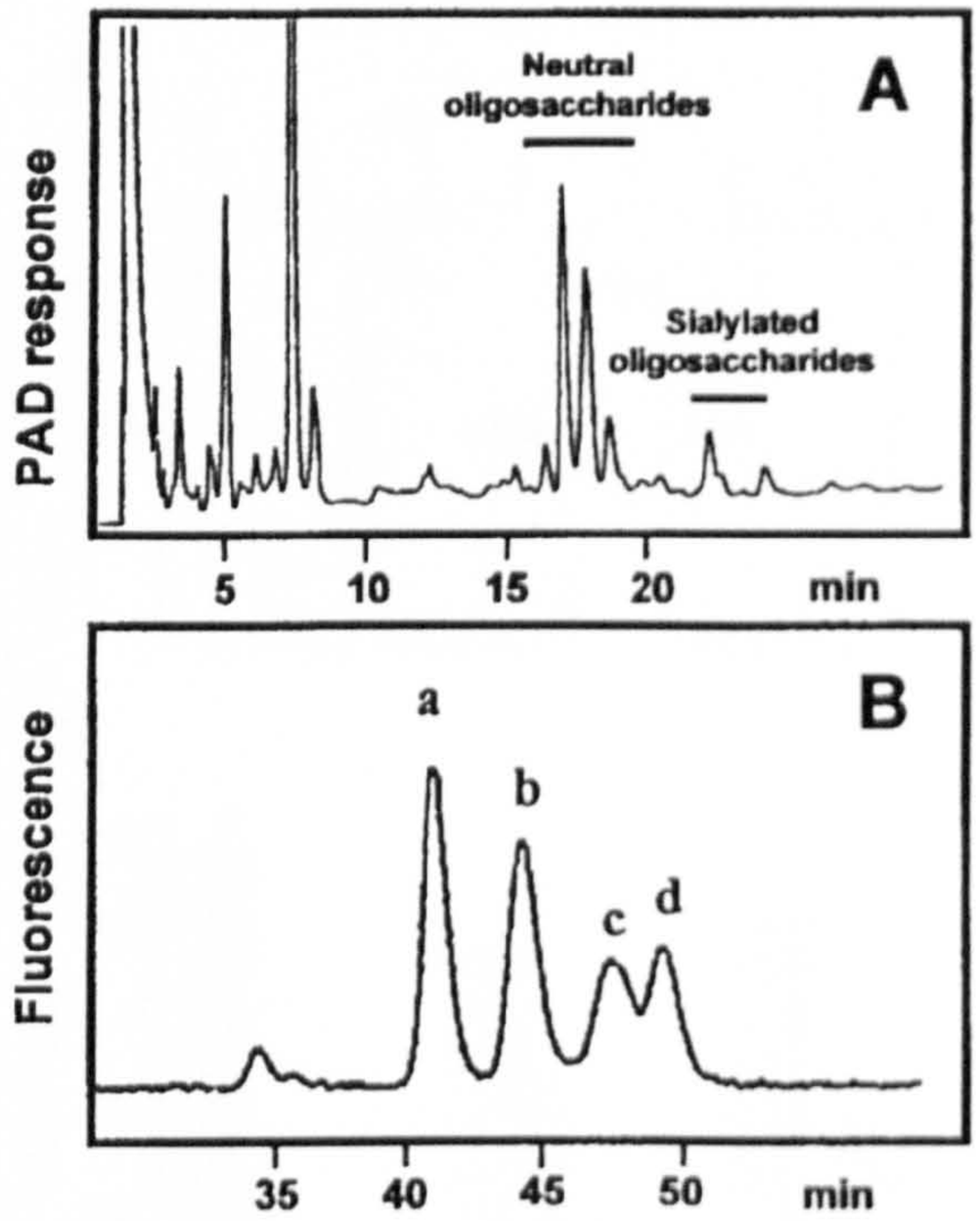


Fig. 2. Chromatography of oligosaccharides released from the mouse MAb Guy's 13. (A) HPAEC-PAD on CarboPac PA1 column of the mouse MAb Guy's 13 N-glycans. (B) HPLC profile on a C18 reverse-phase column of PA-oligosaccharides isolated from the mouse MAb Guy's 13. N-linked glycans released from the mouse MAb with PNGase F were reductively aminated with 2-aminopyridine and separated by reverse-phase HPLC as described in *Materials and methods*. Peaks a to d were identified as the pyridylamino derivatives of oligosaccharides A to D, respectively, presented in Table I.

oligosaccharides (PA-oligosaccharides) were chromatographed by HPLC on a C18 reverse-phase column (Figure 2B). Mostly four neutral oligosaccharides were detected and identified as the pyridylamino derivatives of the α(1,6)-fucosylated biantennary glycans A to D represented in Table I by comparison of their retention with previously reported data (Takahashi *et al.*, 1987). The sialylated structures eluted earlier were not analyzed further. The neutral oligosaccharides were separated, and their structure was confirmed by proton NMR. The structure and the degree of sialylation of the N-linked glycans identified from the MAb Guy's 13 are consistent with previously reported N-glycan analysis of mouse IgG (Rademacher *et al.*, 1986; Mizuochi *et al.*, 1987; Rothman *et al.*, 1989; Rohrer *et al.*, 1995).

N-Glycosylation analysis of the plantibody Guy's 13

The plantibody Guy's 13 was purified from mature transgenic tobacco plants by several affinity chromatography steps. A preliminary analysis of the N-linked glycans attached to the plantibody Guy's 13 was obtained by affino- and immunodetection on blots using glycan-specific probes (Figure 3). Evidence for the presence of high-mannose-type N-glycans was deduced from affinodetection with concanavalin A, a lectin which specifically binds to mannose sequences constitutive of Man-5 to Man-9 oligosaccharides (Figure 3, lane 2). Immunoblotting of the

Table I. Structures of neutral N-linked glycans isolated from the mouse MAb Guy's 13

Code	Structure	Mol %
A	GlcNAcβ1-2Manα1 → 6 Manβ1-4GlcNAcβ1-4GlcNAc; 3 → GlcNAcβ1-2Manα1; 6 → Fucα1	37
B	Galβ1-4GlcNAcβ1-2Manα1 → 6 Manβ1-4GlcNAcβ1-4GlcNAc; 3 → GlcNAcβ1-2Manα1; 6 → Fucα1	31
C	GlcNAcβ1-2Manα1 → 6 Manβ1-4GlcNAcβ1-4GlcNAc; 3 → Galβ1-4GlcNAcβ1-2Manα1; 6 → Fucα1	16
D	Galβ1-4GlcNAcβ1-2Manα1 → 6 Manβ1-4GlcNAcβ1-4GlcNAc; 3 → Galβ1-4GlcNAcβ1-2Manα1; 6 → Fucα1	15

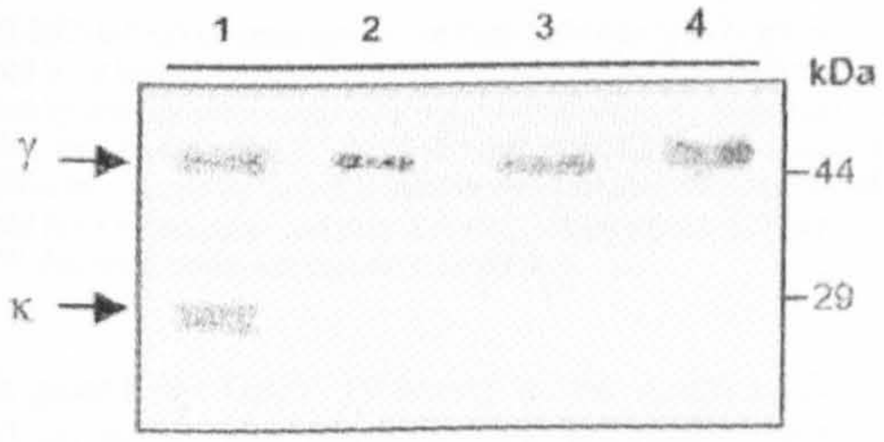


Fig. 3. SDS–PAGE and Western blot analysis of the plantibody Guy's 13. The affinity-purified plantibody Guy's 13 was analyzed by SDS–PAGE in reducing conditions and protein silver staining in the gel (lane 1), or on the corresponding blots by affinodetection with concanavalin A (lane 2) or by immunodetection with antibodies specific for α(1, 3)-fucose (anti-fucose antibodies, lane 3) and for β(1, 2)-xylose (anti-xylose antibodies, lane 4) epitopes of plant N-linked glycans.

plantibody was carried out with antibodies specific for glycan epitopes that are found in plant N-glycans, i.e. antibodies specific for the α(1,3)-fucose residue linked to the proximal glucosamine (anti-fucose antibodies) and antibodies specific for the β(1,2)-xylose residue linked to the β-mannose of the core $Man_3GlcNAc_2$ (anti-xylose antibodies; Faye *et al.*, 1993). The heavy chain was detected on blots with both anti-fucose and anti-xylose antibodies (Figure 3, lanes 3 and 4, respectively). As a consequence, these results indicate that the γ chain of the plantibody Guy's 13 is N-glycosylated by both high-mannose-type N-glycans, and xylose- and fucose-containing complex-type N-glycans. Plant complex-type N-glycans containing Lewis a antigens were recently reported (Fitchette-Lainé *et al.*, 1997; Melo *et al.*, 1997). On blot, the plantibody Guy's 13 was not immunodetected with Lewis a specific antibodies indicating that Lewis a–containing complex-type N-glycans are not attached to the plantibody Guy's 13 (data not shown).

The distribution of plant N-glycans on the two potential N-glycosylation sites of the plantibody Guy's 13 was investigated

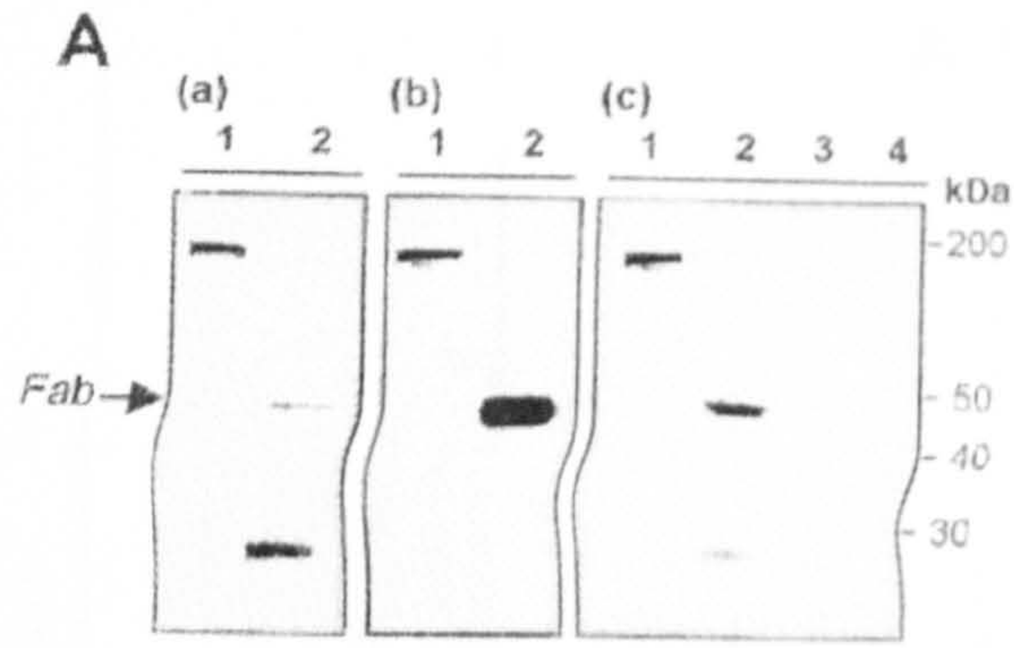


Fig. 4. Immunodetection of complex-type plant N-glycans on Fab and Fc fragments. (A) Immunoblot of plantibody Guy's 13 and papain digested fragments under nonreducing conditions. Three panels are shown, in which detection was with (a) anti-heavy chain, (b) anti-light chain, and (c) anti-xylose antibodies. Lanes 1, undigested plantibody Guy's 13 (4 μg); lanes 2, papain digested plantibody Guy's 13 (4 μg); lane 3, undigested mouse MAb Guy's 13 (4 μg); and lane 4, papain digested mouse MAb Guy's 13 (4 μg). (B) ELISA analysis of the plantibody Guy's 13 and of the corresponding Fab fragment bound to streptococcal antigen. Plantibody Guy's 13 and Fab fragment were incubated in streptococcal antigen coated plates and detected after binding with either anti-heavy chain (anti-Fc fragment) (1:16 dilution), anti-light chain (1:128 dilution) or anti-xylose (1:16 dilution) antibodies. Columns 1, undigested plantibody Guy's 13; columns 2, papain digested plantibody Guy's 13; columns 3, papain digested plantibody purified by incubation with protein G Sepharose beads; columns 4, TBS instead of plantibody or plantibody fragments.

by Western-blot and ELISA analysis (Figure 4). After papain digestion, the resulting fragments were separated in non-reducing conditions and immunodetected on blot with anti-heavy and anti-light chains, as well as with plant glycan-specific antibodies (Figure 4A). Anti-xylose antibodies were found to bind to the Fab fragment of the plantibody on blot (Figure 4A, lane 2c). According to their specificity for plant glycan epitopes, these antibodies do not react with the mouse derived IgG or Fab (Figure 4, lanes 3c and 4c). As a consequence, this result shows that, like the mouse MAb Guy's 13, the plantibody Guy's 13 is N-glycosylated at both sites in the Fc and the Fab. The N-glycosylation of the Fab fragment was confirmed in a functional ELISA. As shown in Figure 4B, the full

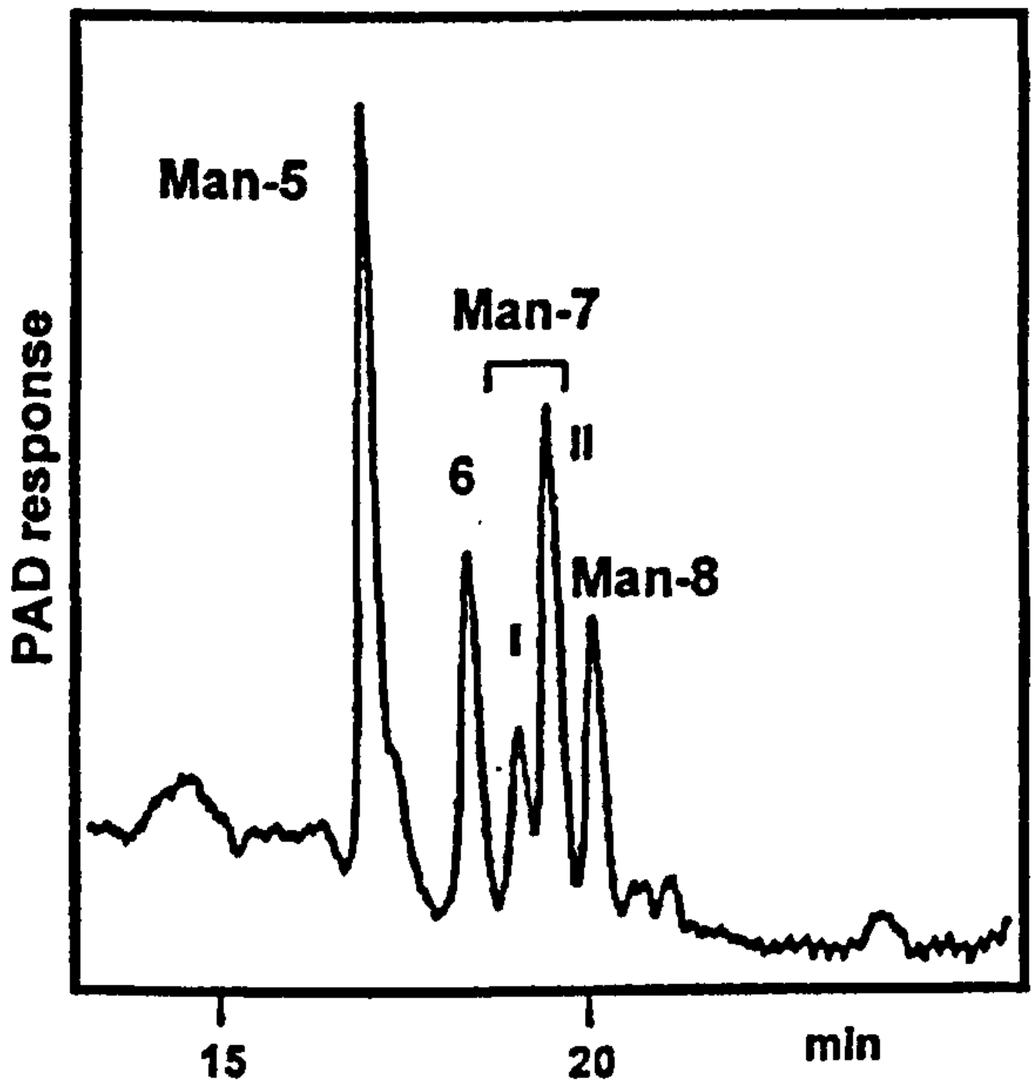


Fig. 5. HPAE-PAD chromatography of high-mannose type N-glycans isolated from the plantibody Guy's 13. High-mannose-type N-glycans were isolated by affinity chromatography on a concanavalin A–Sepharose column and then analyzed by HPAEC-PAD. The different oligosaccharides were identified by comparing their retention times to standard oligosaccharides isolated from mammalian and plant sources. The structures of Man-5 to Man-8 oligosaccharides are presented in Table II.

length plantibody Guy's 13 bound to the streptococcal antigen coated on plates, was recognized by anti-heavy chain (anti-Fc fragment), anti-light chain, and anti-xylose antibodies (Figure 4B, columns 1). Fab fragments produced by papain digest of the plantibody also bind the streptococcal antigen and were still recognized by anti-light chain and anti-xylose antibodies but not by anti-heavy chain (anti-Fc fragment) antibodies (Figure 4B, columns 2). The detection of plant N-glycans on the Fab fragment was confirmed after protein G absorption of the papain digested material to remove intact IgG (Figure 4B, columns 3).

After hydrolysis with 2 M TFA of 50 µg of the plantibody, Fuc, Xyl, GlcNAc, and Man were detected by HPAEC-PAD in the molar ratio 0.5:0.5:3.0:5.0. The glycans N-linked to the heavy chain were released from the whole plant recombinant immunoglobulin by sequential digestion with pepsin and PNGase A as previously reported (Tomiya *et al.*, 1987; Rayon *et al.*, 1996). PNGase A is a plant peptide N-glycosidase which is able to release all N-linked glycans including oligosaccharides having a fucose residue α-linked to the O-3 of the proximal glucosamine. The N-glycans were then purified by elution through a AG50 X2 and a C18 columns, desalted on a Bio Gel P4 column and analyzed by HPAE-PAD chromatography (not shown). The HPAEC-PAD profile has shown a mixture of N-linked glycans which molar ratio were quantified by integrating the amperometric signals detected by HPAEC-PAD. To fully identify the structure of the different oligosaccharides, the mixture of N-glycans released from the plantibody Guy's 13 was fractionated by affinity chromatography on a concanavalin A-Sepharose 4B column in order to separate high-mannose-type N-glycans retained by affinity, from unretained complex-type N-glycans. The fraction containing the high-mannose-type N-glycans was analyzed by HPAE-PAD chromatography (Figure 5). Man-5, Man-6, Man-7(II),

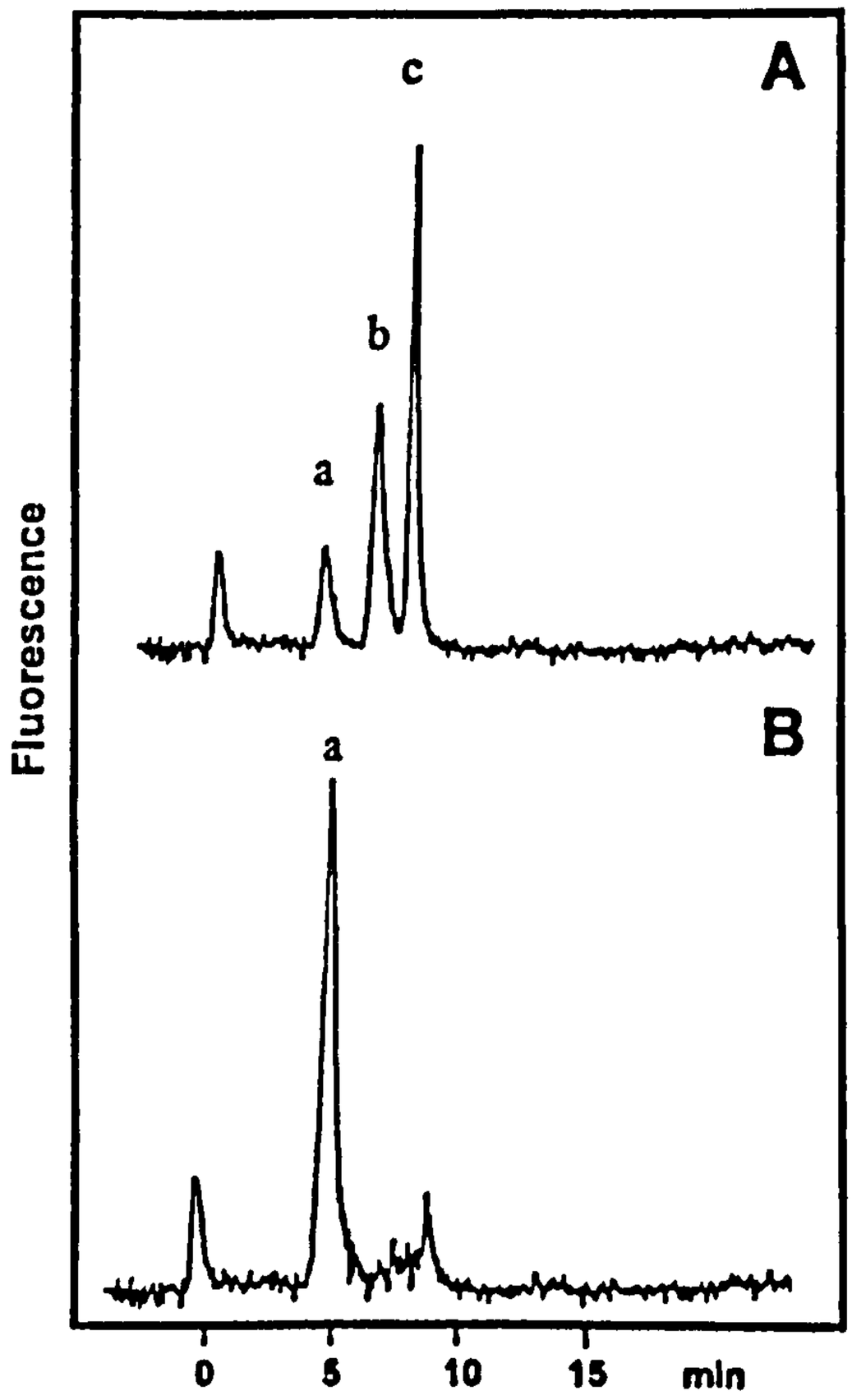


Fig. 6. HPAE chromatography of pyridylamino derivatives of complex-type N-glycans isolated from the plantibody Guy's 13. N-linked glycans that were not retained on immobilized concanavalin A, were coupled with aminopyridine and then analyzed by HPAEC combined to a fluorescence detection. (A) HPAEC profile of PA-oligosaccharides obtained by derivation of N-linked glycans isolated from the plantibody Guy's 13. (B) HPAEC profile of the PA-oligosaccharide obtained after digestion with Jack bean β-N-acetylglucosaminidase. Peaks a, b, and c were identified as the pyridylamino derivatives of oligosaccharides A, B, and C, respectively, presented in Table II.

and Man-8 oligosaccharides were detected and identified by comparing their retention times to those of standard oligosaccharides isolated from mammalian and plant glycoproteins (Rayon *et al.*, 1996; Figure 5, Table II). The structure of the first eluted Man-7 isomer (Man-7(I)) was not fully identified. The oligosaccharides which were not retained by affinity on immobilized concanavalin A were coupled to aminopyridine and then the resulting PA-oligosaccharides were analyzed by HPAE chromatography combined with fluorescence detection as illustrated on Figure 6. Three major peaks—a, b, and c—were detected and identified, respectively, as the pyridylamino derivatives of the plant N-linked glycans A, B, and C presented in Table II, by comparison of their retention times with standard PA-oligosaccharides prepared from plant glycoproteins (Figure 6A). The mixture of labeled glycans was then submitted to an exoglycosidase digestion with jack bean β-N-acetylglucosaminidase. As illustrated in Figure 6B, during the digestion,

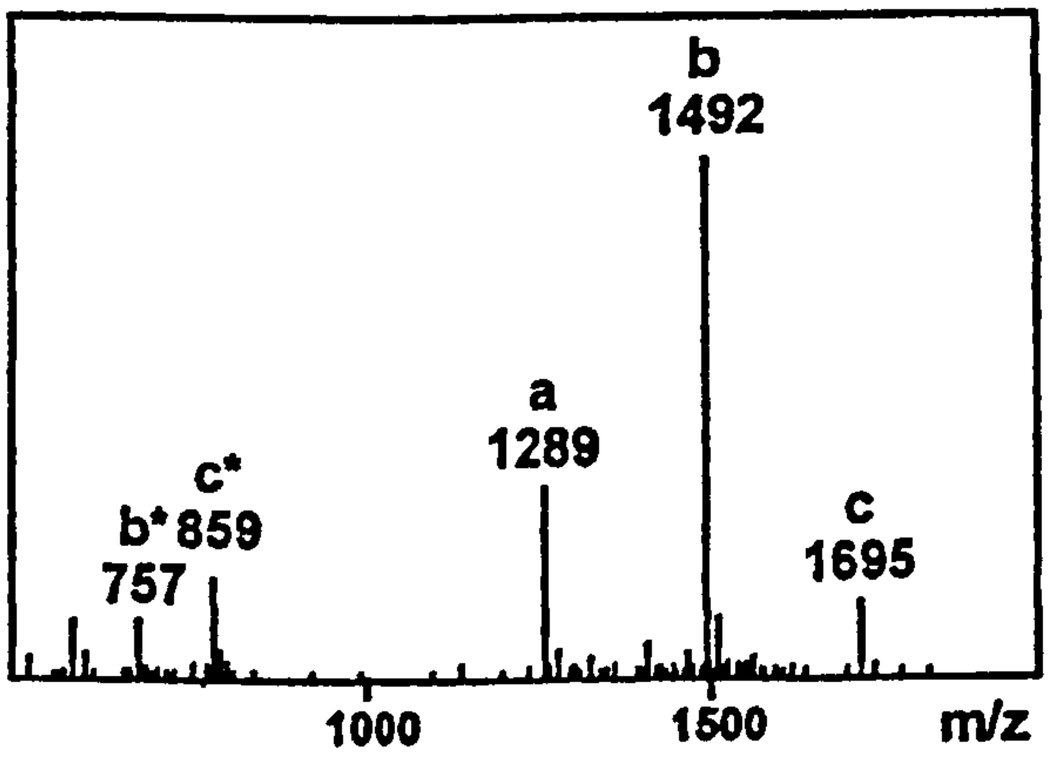
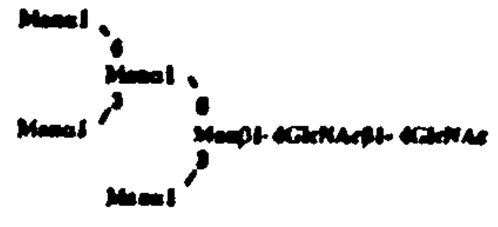
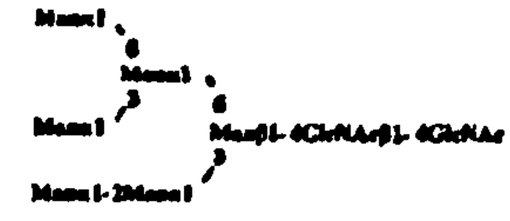
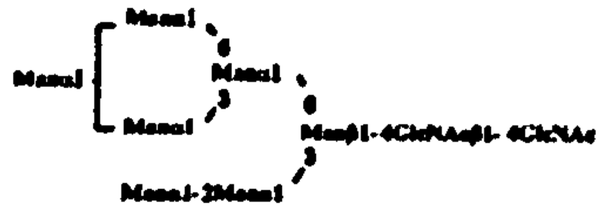
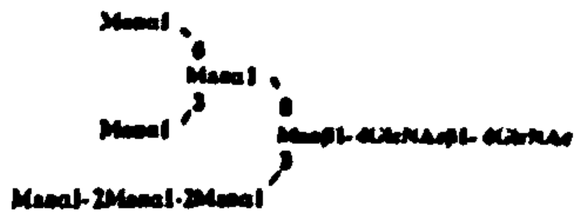
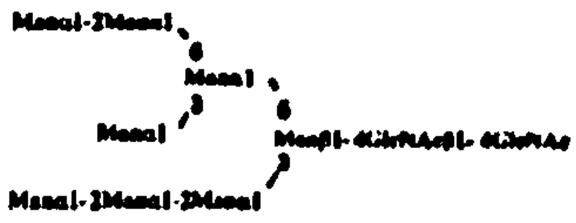
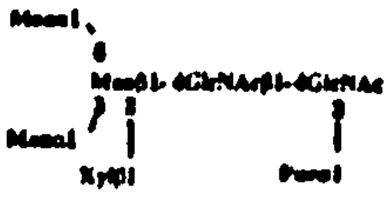
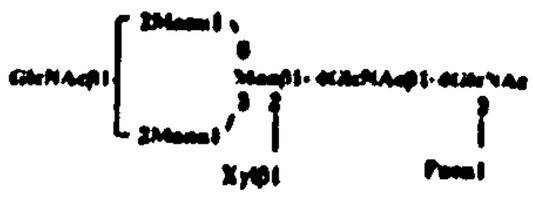
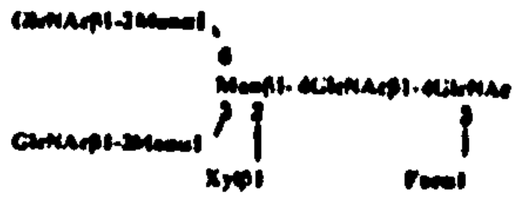


Fig. 7. Electrospray-mass spectrometry of the PA-derivatives of the xylose- and fucose-containing N-glycans isolated from the plantibody Guy's 13. Ions a, b, and c correspond to the $(M+Na)^+$ ions of the pyridylamino derivatives of plant N-glycans A, B, and C presented in Table II. b* and c* are the double charge $(M+2Na)^{2+}$ ions of oligosaccharides B and C.

N-glycans B and C were progressively converted to glycan A as represented in Table II. This result confirms that oligosaccharides B and C eluted in peaks b and c are homologous to oligosaccharide A but have one and two additional terminal GlcNAc residues, respectively (Table II). The structures of complex-type N-glycans associated with the plantibody Guy's 13 were also confirmed by electrospray mass spectrometry (Figure 7). Three ions at m/z = 1289, 1492, and 1695 were detected, which correspond to the adduct ions $(M+Na)^+$ of the pyridylamino derivatives of respectively the complex oligosaccharides A, B, and C, represented Table II.

Table II. Structures of the high-mannose-type (Man-5 to Man-8) and the xylose- and fucose-containing N-glycans A, B and C isolated from the plantibody Guy's 13

Code	Structure	Mol %
Man-5		13
Man-6		9
Man-7(I)		3
Man-7(II)		9
Man-8		6
A		14
B		28
C		18

Discussion

Several mammalian proteins have been successfully produced in plants and some of them are indistinguishable from those produced in mammalian cells as far as amino acid sequence, conformation and eventually biological activity are concerned (Owen and Pen, 1996). However, most clinically important mammalian proteins are glycosylated and their glycosylation affects their physiochemical properties, including resistance to protease attack and solubility. Glycans N-linked to therapeutic glycoproteins are also responsible for some of their biological functions such as antigenicity, immunogenicity, or plasma clearance rate. In a field where the utilization of plants as expression systems for the production of therapeutic recombinant proteins starts to be important, it is urgent to explore the capacity of plant cells to produce and glycosylate mammalian glycoproteins. Our detailed analysis of the N-glycosylation pattern of the MAb Guy's 13, counted with previous results on the biological activity of this MAb expressed in transgenic tobacco (Ma *et al.*, 1994), constitutes the first complete comparative study of mammalian glycoprotein produced in a transgenic plant system.

Four different oligosaccharide structures were found N-linked to the mouse MAb Guy's 13 and were identified as complex-type N-glycans containing α(1,6)-fucose at their innermost N-acetylglucosamine residue and galactose and sialic acid to different extents. These results are consistent with previous structural studies of mouse IgG N-glycans (Rademacher *et al.*, 1986; Mizuochi *et al.*, 1987; Rothman *et al.*, 1989; Rohrer *et al.*, 1995). The N-glycosylation analysis of the plantibody Guy's 13 was carried out on material purified from mature tobacco plants by affinity chromatography on both protein G and immobilized anti-mouse IgG1 antibodies. The diversity of oligosaccharide structures on the plantibody Guy's 13 is far higher than on murine MAb. Indeed, we have identified eight different oligosaccharides on the plantibody molecule representing an array of structurally related oligosaccharides from high-mannose-type N-glycans (40%) to modified glycans (60%). In conclusion, the heterogeneity of the carbohydrate moiety in the antibody Guy's 13 and consequently the number of Guy's 13 glycoforms is higher in transgenic tobacco than in mouse. The plant N-linked glycan structures A, B, and C containing β(1,2)-xylose and the α(1,3)-fucose residues linked to the core $Man_3GlcNAc_2$

(Table II) were previously found in many plant glycoproteins. Furthermore, the complex-type N-glycans B and C, the major oligosaccharides N-linked to the plantibody Guy's 13, are oligosaccharide structures usually found on plant extracellular glycoproteins. These oligosaccharides are known to be rapidly trimmed by the action of exoglycosidases when the glycoprotein is stored in plant vacuole giving the truncated $Man_3XylFucGlcNAc_2$ glycan A (Lerouge *et al.*, 1998). The presence of such oligosaccharides with terminal N-acetylglucosamine residues is indicative of the secretion of most of the plantibody Guy's 13 expressed in transgenic tobacco, which confirms a previous study showing that plantibodies are secreted by plant cells and accumulates in the tobacco apoplasm (Ma and Hein, 1995). However, it cannot be excluded that the large structural heterogeneity of the glycans found on the plantibody could be the result of the storage of part of this recombinant IgG in other compartments of the plant secretory pathway. Indeed, storage of the plantibody in the endoplasmic reticulum or in the vacuole will result in N-glycans having unprocessed or truncated structures, respectively (Lerouge *et al.*, 1998).

The MAb Guy's 13 contains two potential N-glycosylation sites on the heavy chain located on the Fc and the Fab fragments. Both sites of the mouse MAb Guy's 13 were found to be N-glycosylated by complex-type N-glycans. By ELISA and Western blot analysis of the full length or the papain digested plantibody, it was demonstrated that plant complex N-glycans are also localized on both the Fc and the Fab fragments of the plant IgG1. However, the precise distribution of plant N-linked glycans on both sites has not been fully determined, due to the limitations related to the small quantities of purified Fab and Fc fragments isolated from the plantibody. However, our results clearly illustrate that both the highly conserved N-glycosylation site in the C_H2 domain of the heavy chain of murine IgG and the additional site (Asn-74) found on the Fab region of the Guy's 13 are occupied in both the murine and in the plant expression system.

In humans, the mouse MAb Guy's 13 when applied directly to the teeth can prevent colonization of oral cavity with *Streptococcus mutans* and reduce the risk of dental caries (Ma *et al.*, 1990). The production of functional MAb in plants, for a low cost, should provide the large amount of immunoglobulin required for applications in the area of topical immunotherapy. Plant specific glycosylation is sufficient to produce soluble and biologically active IgG. However, the oligosaccharide side chains of immunoglobulins have many other functions than "spacers" between the two C_H2 domains. The IgG N-glycans are necessary for effector functions of the antibodies such as recognition by receptors that mediate their survival in the circulation, binding to macrophage Fc receptors, complement fixation or elimination of antigen–antibody complexes (Wright and Morrison, 1997). Further studies will help to answer whether or not plantibody N-glycans will also assume some of these functions *in vivo*.

Differences that exist in the glycosylation patterns in plants and mammals could represent an important limitation to the use of recombinant mammalian glycoproteins produced in transgenic plants for *in vivo* therapy. In this study, the comparison of murine and plantibody Guy's 13 clearly illustrates these differences in the glycosylation of the same protein backbone. Some similarities exist between complex-type N-glycans from both expression systems such as the presence of a common $Man_3GlcNAc_2$ core substituted by terminal GlcNAc residues. However, additional motifs on this core, i.e., the α(1,3)-fucose and the β(1,2)-xylose residues, can be highly immunogenic in mammals. In the context of large quantities of antibodies that may be introduced into the human organism for some therapies, a sensitization to the plant complex-type N-glycans identified on the plantibody Guy's 13, might occur. As a consequence, for some *in vivo* applications of recombinant antibodies produced in transgenic plants, strategies have to be developed to obtain plantibodies with carbohydrate profiles structurally more consistent with ones obtained from mammalian cells.

Materials and methods

Materials

Ricinus communis agglutinin (RCA) coupled with horseradish peroxidase, pepsin and β-N-acetylglucosaminidase from Jack bean were from Sigma. PNGase A and F were purchased from Boehringer Mannheim. Man-5 (RNase B), Man-6 (RNase B), Man-7(II) (bean phaseolin), Man-8 (bean phytohemagglutinin), and Man-9 (soybean agglutinin) were isolated from mammalian and plant glycoproteins and identified by proton NMR. The pyridylamino derivative standards of plant N-glycans were prepared by labeling with 2-aminopyridine (Hase *et al.*, 1984) complex oligosaccharides isolated from bean phytohemagglutinin (Rayon *et al.*, 1996) and from *Arabidopsis thaliana* glycoproteins.

Purification of the plantibody Guy's 13

Mature transgenic plants expressing Guy's 13 MAb were homogenized in 1.5 volume of Tris-buffered saline (TBS, 75 mM NaCl and 10 mM Tris–HCl, pH 8). After centrifugation of the homogenate at 12,000 × *g* for 30 min at 4°C, proteins were precipitated from the supernatant in the presence of ammonium sulfate (50% saturation). After a second centrifugation in the same conditions as above, the protein pellet was solubilized in TBS. The proteins were then submitted to a second precipitation with ammonium sulfate (50% saturation). The second protein precipitate was resuspended in TBS and the solution was passed through a 0.45 µm filter. IgG was purified from this protein extract by affinity chromatography on a protein G–agarose column (MAbtrap GII, Pharmacia Biotech) followed by a further affinity purification step using Sepharose 4B beads coupled to goat anti-mouse IgG1 antibodies (Sigma UK).

SDS–PAGE and immunoblotting experiments

The antibody Guy's 13 was separated in a 15% SDS-PAGE in both reducing and nonreducing conditions and was then transferred onto a nitrocellulose membrane. Immunodetections with anti-xylose or anti-fucose antibodies were performed according to Faye *et al.* (1993). Affinodetection of high-mannose-type N-glycans was carried out using the concanavalin A/peroxidase method (Faye and Chrispeels, 1985). Affinodetection by RCA was carried out as reported in Fitchette-Lainé *et al.* (1998).

Papain digestion of plantibody Guy's 13

Purified plantibody Guy's 13 was incubated with papain (1:100) (Sigma, UK) in 0.1 M Tris–HCl pH 7 containing 2 M EDTA and 20 mM cysteine, for 16 h at 37°C. The reaction was stopped by incubation with 0.1 M iodoacetamide for 1 h at 37°C. Contaminating Fc was removed by incubation with protein G Sepharose beads (Pharmacia Biotech, UK) for 5 h at 37°C, and the supernatant was recovered by centrifugation.

ELISA analysis of plantibody and Fab fragment bound to streptococcal antigen

ELISA plates (Dynatech, Immulon) were coated with streptococcal antigen at a predetermined optimal concentration of 2 µg/ml. After blocking with 5% nonfat dry milk in TBS containing 0.01% Tween 20, plantibody solutions were applied for 2 h at 37°C. The plates were washed with TBS containing 0.05% Tween 20, and either anti-murine kappa (Bradsure Biologicals, UK), anti-murine gamma (anti-Fc fragment) (Sigma, UK) or anti-xylose antibodies were applied for 2 h at 37°C. After further washings, the next incubation was done with an appropriate alkaline phosphatase–conjugated antiserum for 2 h at 37°C. Finally, detection was performed using disodium p-nitrophenylphosphate (Sigma, UK). Absorbance was read at 405 nm.

Monosaccharide analysis

Ten micrograms of mouse or plantibody Guy's 13 were hydrolyzed with 2 M TFA at 105°C for 2 h. The samples were dried out, and the analysis of monosaccharides was carried out by HPAEC-PAD as previously reported (Hardy, 1989). Sialic acids were released from the mouse MAb with 0.2 M TFA at 80°C for 1 h. The analysis and quantification of the sialic acids was done by HPAEC-PAD according to Anumula and Taylor (1991).

Isolation of N-linked glycans from the mouse MAb

The purified mouse Guy's 13 (3 mg) was digested with trypsin (1% w/w) in a 50 mM ammonium carbonate buffer, pH 8, for 18 h at 37°C. The solution was then heated at 100°C for 5 min and treated with PNGase F (10 mU) for 24 h at 37°C. The oligosaccharides were purified by successively passing through a 2 ml AG50 X2 column and a C18 Sep-Pak cartridge. The oligosaccharides were finally desalted on a 40 × 1 cm Bio Gel P4 column.

Isolation of N-linked glycans from the plantibody

N-linked glycans were released from 2 mg of purified plantibody Guy's 13 by sequential digestions with pepsin and PNGase A (1 mU) as previously reported (Tomiya *et al.*, 1987; Rayon *et al.*, 1996). The resulting free glycans were desalted on a Bio Gel P4 column. Complex-type N-glycans were then separated from the high-mannose-type N-glycans by affinity chromatography on a concanavalin A-Sepharose 4B column as reported previously (Montreuil *et al.*, 1986).

Analysis of N-linked glycans

HPAE-PAD chromatographies of oligosaccharides released from the mouse MAb and high-mannose-type N-glycans isolated from the plantibody were achieved on a Dionex DX 500 system equipped with a GP 50 gradient pump and a CarboPac PA1 column. Oligosaccharides were separated using a linear gradient from 0 to 100 mM NaOAc in 100 mM NaOH at 1 ml/min over 30 min. High-mannose-type N-glycans from the plant MAb were identified by comparing their retention times to standard oligosaccharides isolated from RNase B (Man-5 and Man-6), bean phaseolin (Man-7(II)), bean phytohemagglutinin (Man-8), and soybean agglutinin (Man-9) as previously reported (Rayon *et al.*, 1996). Complex-type N-glycans were coupled with aminopyridine as described by Hase *et al.* (1984). Pyridylamino derivatives (PA) of the N-linked glycans isolated from the mouse MAb were separated by reverse-phase HPLC on a Spherisorb ODS 2 C18 column (250 × 4.6 mm). Elution and identification of PA-oligosaccharides were performed as described in Tomiya *et al.* (1988). The quantification of the molar ratio of the N-linked glycans isolated from the mouse MAb was deduced by integrating the fluorescence signals of PA-derivatives separated by reverse-phase HPLC. HPAE chromatography of the PA-derivatives of complex-type N-glycans isolated from the plantibody was achieved as described above for the separation of reducing oligosaccharides. However, PA-oligosaccharides were detected using a Spectra System FL 2000 (Spectra-Physics) fluorescence detector with excitation and emission wavelength at 320 and 400 nm, respectively. The sample was dissolved in 0.1 M NaOH before injection. Exoglycosidase treatment of the PA-oligosaccharides was carried out with 1 U of β-N-acetylglucosaminidase in a 10 mM NaOAc buffer, pH 5, at 25°C for 18 h. The quantification of the molar ratio of the N-linked glycans isolated from the plantibody was assessed by integrating the amperometric signals detected by HPAEC-PAD of the mixture of complex and high-mannose-type N-glycans.

Electrospray mass ionization

Electrospray mass ionization was performed on a quadrupolar mass spectrometer NERMAG R 1010U equipped with an Analytica of Branford atmospheric pressure electrospray source (Quad Servive, Poissy, France). The mass on charge (*m/z*) range was of 2000. The sample was dissolved in a 1:1 H_2O:CH_3OH solution containing 0.05% CH_3COOH and infused into the electrospray ion source at a flow-rate of 1.5 µl/min by a Harvard syringe pump (Harvard Apparatus). The ion source temperature was 80°C (drying gas). Full scan spectra were acquired in the range of 500–1950 at a scan speed of 9s.

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Abbreviations

HPAEC-PAD, high pH anion exchange chromatography coupled to pulsed amperometric detection; MAb, monoclonal antibody; ^{1}H NMR, proton nuclear magnetic resonance; PA, pyridylamino derivative; plantibody, recombinant antibody produced in transgenic plants; PNGase A, peptide N-glycosidase A; PNGase F, peptide N-glycosidase F; RCA, *Ricinus communis* agglutinin.

References

Anumula,K.R. and Taylor,P.B. (1991) Rapid characterization of asparagine-linked oligosaccharides isolated from glycoproteins using a carbohydrate analyser. *Eur. J. Biochem.*, 195, 269–280.

During,K., Hippe,S., Kreuzaler,F. and Schell,J. (1990) Synthesis and self-assembly of a functional monoclonal antibody in transgenic *Nicotiana tabacum*. *Plant Mol. Biol.*, 15, 281–293.

Faye,L. and Chrispeels,M.J. (1985) Characterization of N-linked oligosaccharides by affinoblotting with concanavalin A-peroxidase and treatment of the blots with glycosidases. *Anal. Biochem.*, 149, 218–224.

Faye,L., Gomord,V., Fitchette-Lainé,A-C. and Chrispeels,M.J. (1993) Affinity purification of antibodies specific for Asn-linked glycans containing α 1–3 fucose or β 1–2 xylose. *Anal. Biochem.*, 109, 104–108.

Fitchette-Lainé,A-C., Gomord,V., Cabanes,M., Saint Macary,M., Foucher,B., Michalski,J-C., Hawes,C., Lerouge,P. and Faye,L. (1997) N-Glycans harboring the Lewis a epitope are expressed at the surface of plant cells. *Plant J.*, **12**, 1411–1417.

Fitchette-Lainé,A-C., Denmat,L-A., Lerouge,P. and Faye,L. (1998) Analysis of N- and O-glycosylation of plant proteins. *Methods Biotechnol.*, **3**, 271–290.

Hase,S., Ibuki,T. and Ikenaka,T. (1984) Reexamination of the pyridylamination used for fluorescence labeling of oligosaccharides and its application to glycoproteins. *J. Biochem.*, **95**, 197–203.

Hardy,M.R. (1989) Monosaccharide analysis of glycoconjugates by high-performance anion-exchange chromatography with pulsed amperometric detection. *Methods Enzymol.*, **179**, 76–82.

Hiatt,A.C., Cafferkey,R. and Bowdish,K. (1989) Production of antibodies in transgenic plants. *Nature*, **342**, 76–78.

Jefferis,R. and Lund,J. (1997) Glycosylation of antibody molecules: structural and functional significance. *Antibody Eng. Chem. Immunol.*, **65**, 111–128.

Lerouge,P., Cabanes-Macheteau,M., Rayon,C., Fitchette-Lainé,A-C., Gomord,V. and Faye,L. (1998) N-Glycoprotein biosynthesis in plants: Recent developments and future trends. *Plant Mol. Biol.*, **38**, 31–48.

Ma,J.K.-C. and Hein,M.B. (1995) Immunotherapeutic potential of antibodies produced in plants. *Trends Biotechnol.*, **13**, 522–527.

Ma,J.K.-C., Hunjan,M., Smith,R. Kelly,C. and Lehner,T. (1990) An investigation into the mechanism of protection by local passive immunisation with monoclonal antibodies against *Streptococcus mutans*. *Infect Immunol.*, **58**, 3407–3414.

Ma,J.K.-C., Lehner,T., Stabila,P., Fux,C.I. and Hiatt,A. (1994) Assembly of monoclonal antibodies with IgG1 and IgA heavy chain domains in transgenic tobacco plants. *Eur. J. Immunol.*, **24**, 131–138.

Ma,J.K.-C., Hiatt,A., Hein,M.B., Vine,N.D., Wang,F., Stabila,P., van Dolleweerd,C., Mostov,K. and Lehner,T. (1995) Generation and assembly of secretory antibodies in plants. *Science*, **268**, 716–719.

Melo,N.S., Nimtz,M., Conradt,H., Fevereiro,P.S. and Costa,J. (1997) Identification of the human Lewis^a carbohydrate motif in a secretory peroxidase from a plant cell suspension culture (*Vaccinium myrtillus L.*). *FEBS Lett.*, **415**, 186–191.

Mizuochi,T., Hamako,J. and Titani,K. (1987) Structure of the sugar chains of mouse immunoglobulin G. *Arch. Biochem. Biophys.*, **257**, 387–394.

Moffat,A.S. (1995) Exploring transgenic plants as a new vaccine source. *Science*, **268**, 658–660.

Montreuil,J., Bouquelet,S., Debray,H., Fournet,B., Spik,G. and Strecker,G. (1986) Carbohydrate analysis: a pratical approach. In Chaplin,M.F. and Kennedy,J.F (eds), *Carbohydrate Analysis: A Practical Approach.*, IRL Press, Oxford, pp. 143–204

Owen,M.R.L. and Pen,J. (1996) *Transgenic Plants: A Production System for Industrial and Pharmaceutical Proteins*. John Wiley and Sons, Chichester.

Parekh,R.B., Dwek,R.A., Sutton,B.J., Fernandes,D.L., Leung,A., Stanworth,D., Rademacher,T.W., Mizuochi,T., Taniguchi,T., Matsuta,K., Takeuchi,F., Nagano,Y., Miyamoto,T. and Kobata,A. (1985) Association of rheumatoid arthritis and primary osteoarthritis with changes in the glycosylation pattern of total serum IgG. *Nature*, **316**, 452–457.

Rademacher,T.W., Homans,S.W., Parekh,R.B. and Dwek,R.A. (1986) Immunoglobulin G as glycoprotein. *Biochem. Soc. Symp.*, **51**, 131–148.

Rayon,C., Gomord,V., Faye,L. and Lerouge,P. (1996) N-Glycosylation of phytohemagglutinin expressed in bean cotyledons or in transgenic tobacco plants. *Plant Physiol. Biochem.*, **34**, 273–281.

Rohrer,J., Thayer,J., Avdalovic,N. and Weitzhandler,M. (1995) HPAEC-PAD analysis of monoclonal antibodiy glycosylation. *Techniques Protein Chem.*, **6**, 65–73.

Rothman,R.J., Warren,L., Vliegenthart,J.F.G. and Hard,K.J. (1989) Clonal analysis of the glycosylation of immunoglobulin G secreted by murine hybridomas. *Biochemistry*, **28**, 1377–1384.

Smith,R. and Lehner,T. (1989) Characterisation of monoclonal antibodies to common protein epitopes on the cell surface of *Streptococcus sobrinus*. *Oral Microbiol. Immunol.*, **4**, 153–158.

Takahashi,N., Ishii,I., Ishihara,H., Mori,M., Tejima,S., Jefferis,R., Endo,S. and Arata,Y. (1987) Comparative structural study of the N-linked oligosaccharides of human normal and pathological immunoglobulin G. *Biochemistry*, **26**, 1137–1144.

Tomiya,N., Kurono,M., Ishihara,H., Tejima,S., Endo,S., Arata,Y. and Takahashi,N. (1987) Structural analysis of N-linked oligosaccharides by a combination of glycopeptidase, exoglycosidases, and high-performance liquid chromatography. *Anal. Biochem.*, **163**, 489–499.

Tomiya,N., Awaya,J., Kurono,M., Endo,S., Arata,Y. and Takahashi,N. (1988) Analyses of N-linked oligosaccharides using a two-dimensional mapping technique. *Anal. Biochem.*, **171**, 73–90.

Wright,A. and Morrison,S.L. (1997) Effect of glycosylation on antibody function: implications for genetic engineering. *Trends Biotechnol.*, **15**, 26–32.

Characterization of a recombinant plant monoclonal secretory antibody and preventive immunotherapy in humans

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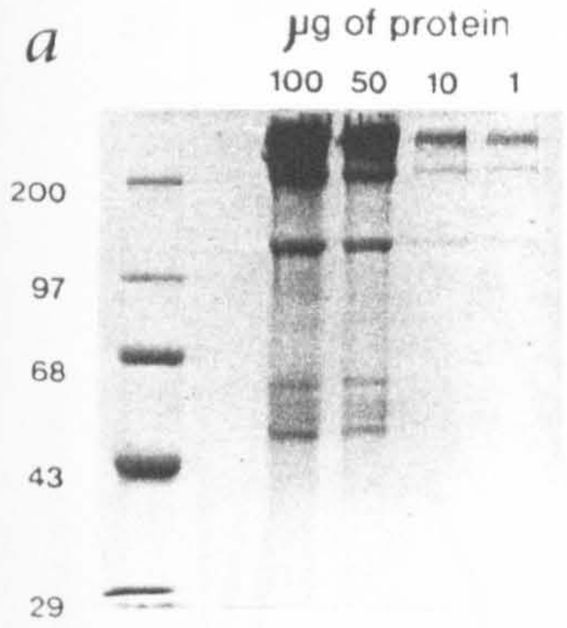
A functional comparison was made between a monoclonal secretory antibody generated in transgenic plants and its parent murine IgG antibody. The affinity constants of both antibodies for a *Streptococcus mutans* adhesion protein were similar. However the secretory antibody had a higher functional affinity due to its dimeric structure. In the human oral cavity, the secretory antibody survived for up to three days, compared with one day for the IgG antibody. The plant secretory antibody afforded specific protection in humans against oral streptococcal colonization for at least four months. We demonstrate that transgenic plants can be used to produce high affinity, monoclonal secretory antibodies that can prevent specific microbial colonization in humans. These findings could be extended to the immunotherapeutic prevention of other mucosal infections in humans and animals.

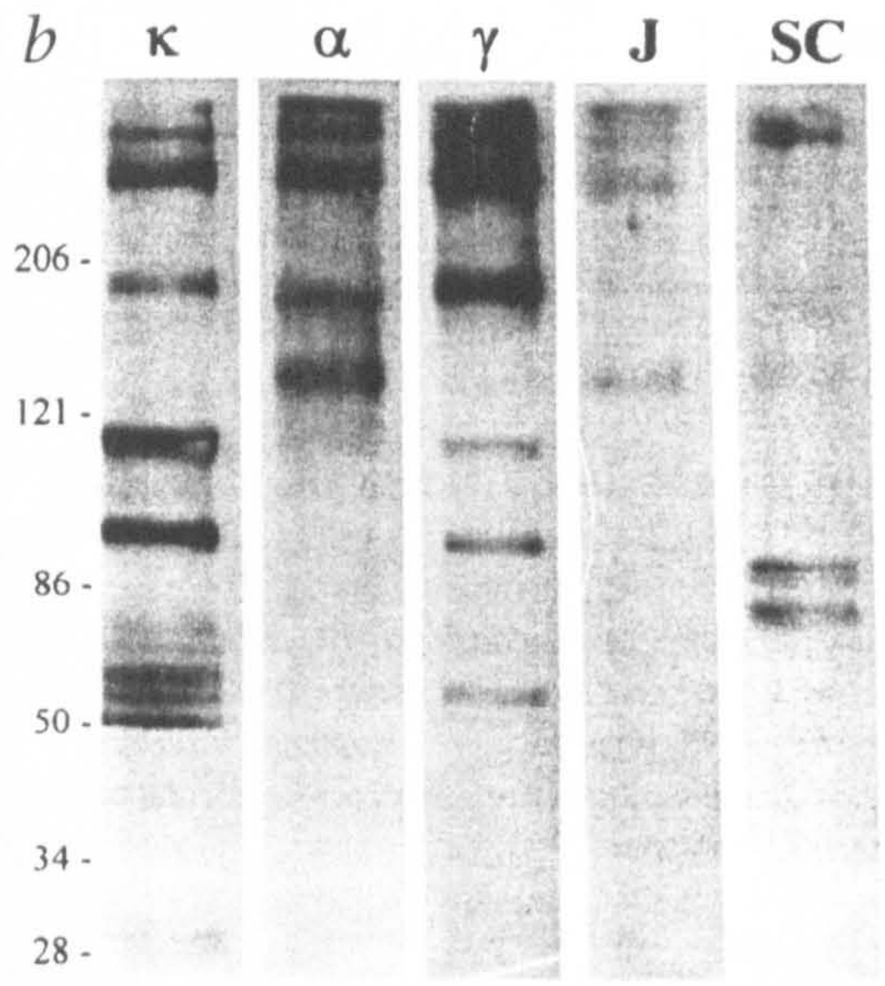
Plants are increasingly being used for the production of recombinant immunotherapeutic agents[1]. An important advantage is the fidelity with which plants can express, fold, assemble and process foreign proteins. Moreover, there is a potentially significant cost benefit in growing bulk quantities of recombinant proteins in plants. Complex multimeric proteins such as IgG and secretory antibodies can be produced with high efficiency in transgenic plants[2,3], presenting an opportunity for passive immunotherapy, particularly at mucosal surfaces where large quantities of recombinant antibody would be required. We have previously demonstrated that topical application of two IgG monoclonal antibodies (mAbs) that recognise the 185-Kd cell surface adhesion protein of *Streptococcus mutans* specifically prevents bacterial colonization and the development of dental caries in non-human primates[4]. Prevention of colonization of *S. mutans* in humans has been demonstrated using the same IgG mAbs[5,6]. We have also described the engineering and expression of a secretory form of one of these antibodies in transgenic plants[3]. In this molecule, α-chain domains from an IgA mAb were engineered into the constant region of mAb Guy's 13 (ref. 7), which allowed dimerization with J chain and assembly with secretory component. The current study was designed to compare secretory antibody (SIgA/G) and the original mouse IgG versions of Guy's 13, with regard to *in vitro* function and *in vivo* activity, and prevention of bacterial colonization in humans.

Purification of recombinant secretory antibody

Constructs encoding the four proteins comprising Guy's 13 SIgA/G have been described[3]. Briefly, the heavy chain consisted of mouse γ1 and α domains, the κ light chain and J chain were also murine, and the secretory component was rabbit in origin. Recombinant SIgA/G was purified from mature tobacco plants by ammonium sulfate precipitation and protein G affinity chromotography. The yield of antibody was between 10–80 mg/kg fresh weight of plant material. The final preparation was in sterile phosphate buffered saline (PBS), pH 7, at a total protein concentration of 10 mg/ml. Endotoxin contamination was less than 0.1 endotoxin units/ml.

On SDS-PAGE analysis, the purified antibody preparation consists of four major protein bands (Fig. 1*a*). The most abundant proteins have the highest molecular weights, which correspond to SIgA/G and dimeric IgA/G respectively (Fig. 1*b*). Densitometric analyses suggested that these two species represent at least 70% of the total protein and that the SIgA/G constitutes more than half of this. The third major band runs alongside the M_r 200K protein marker and represents monomeric IgA/G (Fig. 1*a*). The fourth major band has an approximate M_r of 110K and corresponds to the $F(ab')_2$ proteolytic fragment. The identity of these bands was determined by comparison with a western blot of crude plant extracts using specific antisera against the different components of the SIgA/G molecule, which demonstrates all of the immunoglobulin related molecules present (Fig. 1*b*). There were five additional minor protein bands between M_r 43–68K in the purified SIgA/G preparation (Fig. 1*a*) which probably represent free secretory component (SC) and breakdown products of the antibody preparation. SIgA can occur *in vivo* as a mixture of covalently and noncovalently linked molecules, which may explain the presence of free SC in the protein immunoblot analysis. In addition, plants produce a number of proteases which might account for the fragmentation of immunoglobulin molecules during processing...





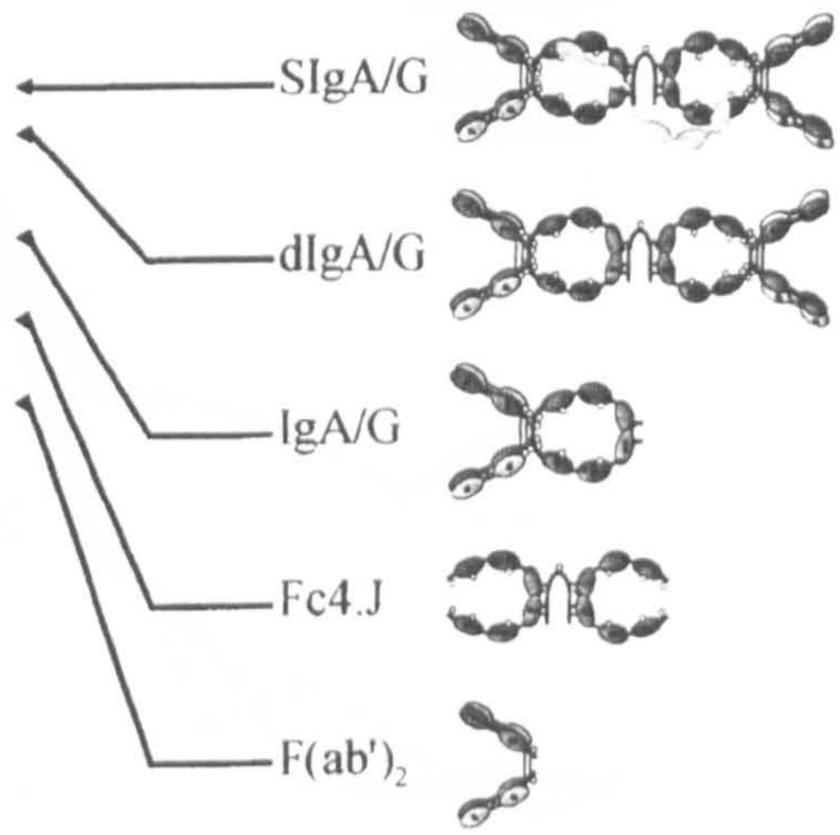


Fig. 1 *a*, SDS - PAGE of the purified plant SIgA/G preparation. Four loading amounts of protein are shown. *b*, Western blot analysis of recombinant plant proteins. Immunoblots of unpurified plant extract separated on a 5–18% tris-tricine gradient gel are shown. The antiserum used for detection of each blot is indicated, as are the major bands and their deduced structures.

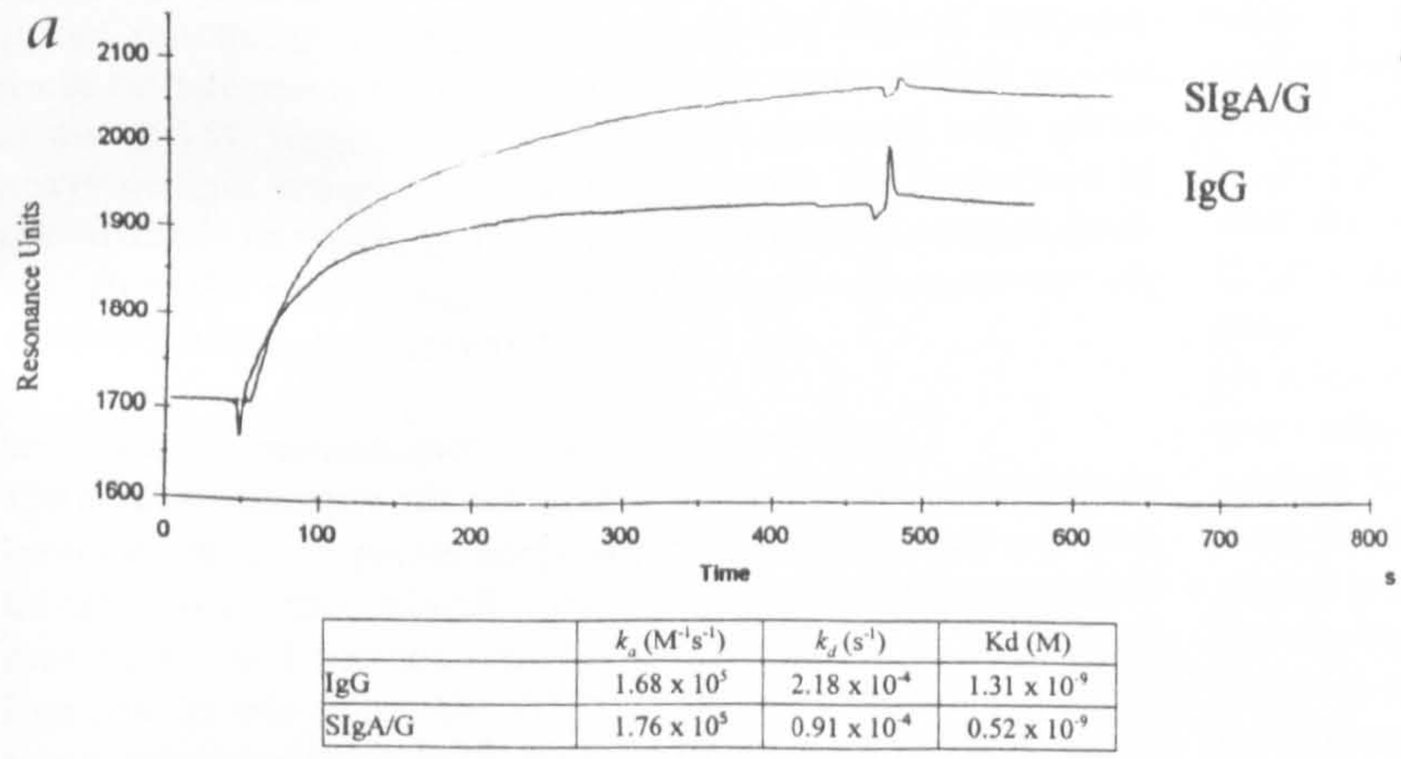
Affinity and avidity of SIgA/G and IgG

The binding affinity of the plant SIgA/G to streptococcal antigen I/II was compared with that of the native mouse IgG Guy's 13 by surface plasmon resonance. The binding curves for both forms of antibody at equimolar concentrations were similar for both the association and dissociation phases. A representative sensorgram is shown (Fig. 2*a*). The values for k_a, k_d and Kd were calculated from sensorgrams using five concentrations for each antibody. There was no difference between the deduced dissociation constants for plant SIgA/G (Kd = 5.2×10^{-10}M) and IgG (Kd = 1.3×10^{-9}M) monoclonal antibodies. This assay is optimized for a 1:1 molecular interaction, so it is unlikely that differences in avidity would be apparent. However, the Biacore analysis does serve to confirm the correct assembly of the antigen binding site of the antibody in plants and demonstrates that antigen recognition is unaffected by any differences in antibody structure, due to the complex nature of the SIgA/G molecule or variations between plant and murine glycosylation.

A comparison of the functional affinities of the two antibody preparations was determined using a competition ELISA, in which, by addition of antigen in the fluid phase, the antigen is in excess to favor multivalent interactions with antibody. Approximately 4-fold higher antigen concentration was required to inhibit binding of IgG to SA I/II by 50% (IC_{50} = ~43 nM), compared with the plant SIgA/G (IC_{50} = ~11 nM) (Fig. 2), which demonstrates that the SIgA/G preparation had a higher functional affinity than IgG. As the dissociation constants of the two antibodies are the same, this difference can be accounted for by the higher avidity of the tetravalent SIgA/G and dIgA/G molecules compared with the bivalent IgG molecule[8].

Comparative survival of antibodies *in vivo*

It has been suggested that secretory antibodies might survive longer than serum antibodies in the mucosal environment due



	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	Kd (M)
IgG	1.68×10^5	2.18×10^{-4}	1.31×10^{-9}
SIgA/G	1.76×10^5	0.91×10^{-4}	0.52×10^{-9}

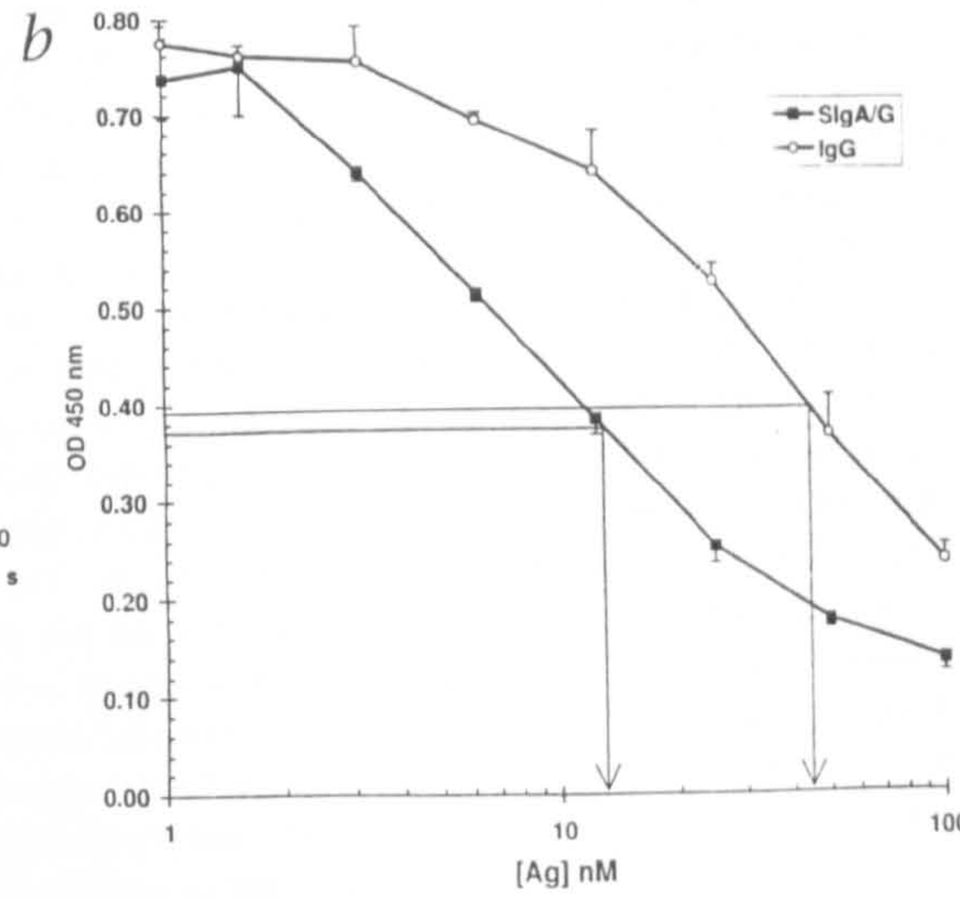


Fig. 2 *a*, Antibody affinity measurement by surface plasmon resonance. A representative sensorgram for SIgA/G is shown superimposed on that for IgG (both at 87.5 nM concentration), to illustrate association and dissociation phases. The *ka, kd* and Kd values were determined from five sensorgrams using antibody at concentrations between 50–100 nM. *b*, Antibody avidity measurement by competition ELISA. Values shown are mean ± standard deviation of triplicate samples. The relative avidities of the SIgA/G and IgG MAbs are determined by calculating the concentration of free antigen required to inhibit antibody binding by 50% (IC_{50}).

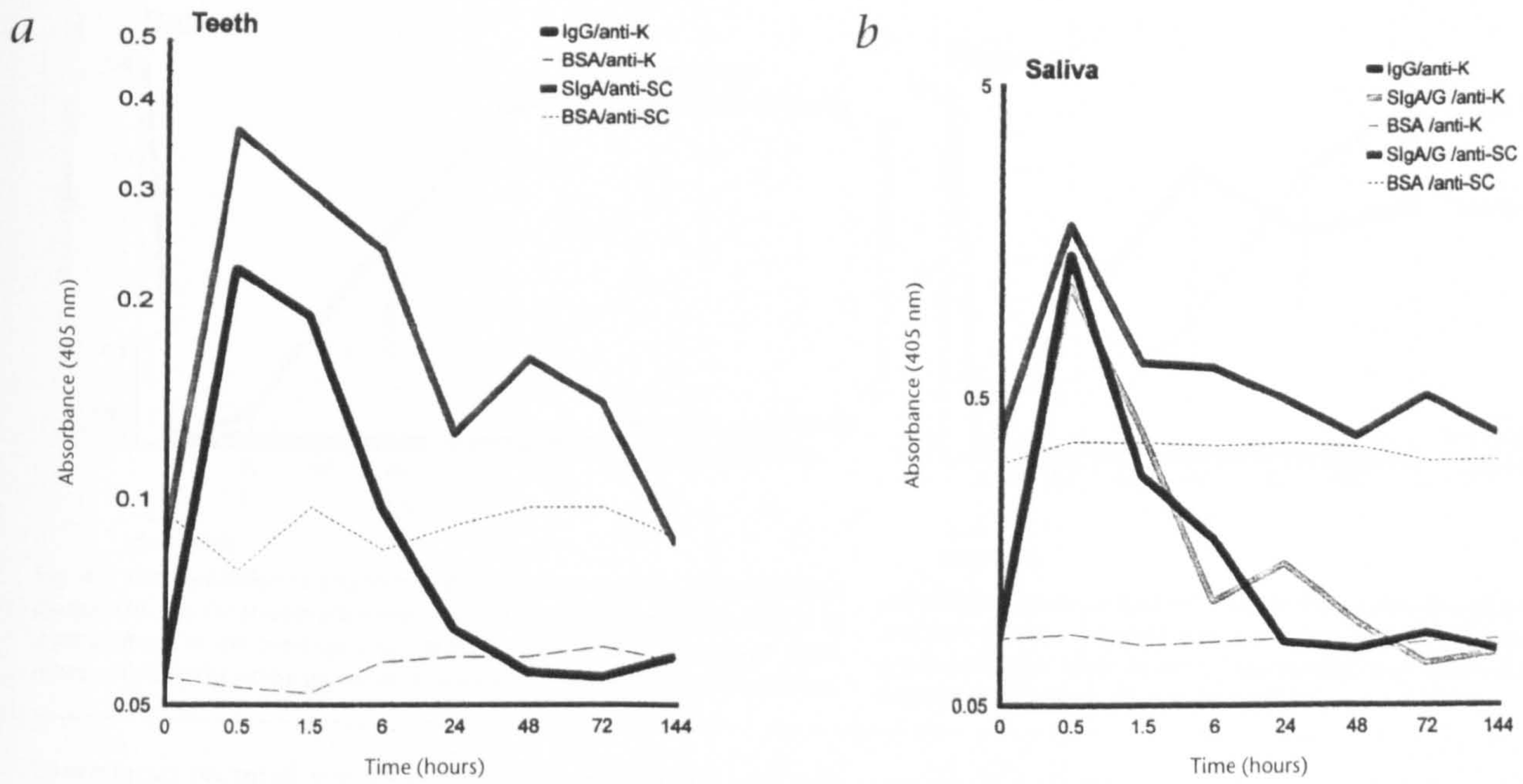


Fig. 3 Duration of topically applied mouse Guy's 13 IgG and plant SIgA/G *in vivo*. Representative data from one patient in each group are shown. Detection of recoverable antibody was by antigen specific ELISA, using either an anti-light chain or anti-SC reagent and the results shown are the mean of triplicate samples., Detection of Guy's 13 antibody from teeth (*a*) and saliva (*b*). Samples from the control subject (BSA) are shown as dotted lines and demonstrate the baseline detection limits using either anti-kappa or anti-SC detection antisera. Samples from the subject given SIgA/G are shown as red solid lines and those from the subject given IgG as blue solid lines.

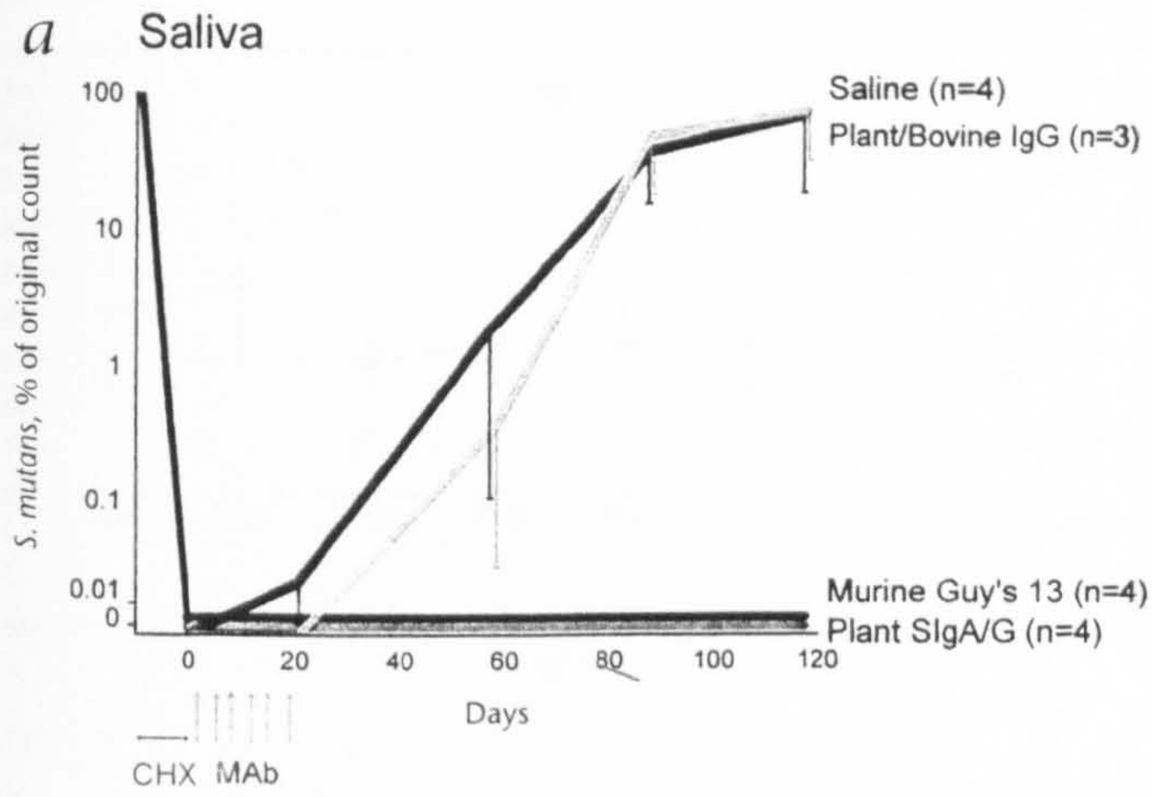
to the presence of the secretory component which protects the molecule against proteolysis[9–11]. We have investigated this directly *in vivo* by examining the survival of the two forms of Guy's 13 antibodies applied topically into the oral cavity. Six human volunteers were used and representative data are shown (Fig. 3). The baseline limits of detection were determined using control subjects who were given bovine serum albumin in PBS by the same method as that used for the mAbs. Following the application of mAb solution to the teeth, both antibodies were detectable after 30 minutes. In subjects who received IgG, the recoverable antibodies on teeth and in saliva then decreased rapidly and were undetectable after 24 hours. In contrast, SIgA/G was detectable for at least 72 hours on teeth (Fig. 3*a*) and for up to 48 hours in saliva (Fig. 3*b*). The detection assay required functional antigen binding and the SIgA/G antibody could be detected with both an anti-light chain reagent as well as an anti-SC reagent which does not crossreact with either heavy or light chains[3]. SC is assembled with the Fc portion of the antibody molecule and does not contribute to antigen binding, thus the results suggest that the antibody recovered remained intact in the oral cavity.

Local passive immunization in human volunteers

The clinical protective efficacy of mAb Guy's 13 IgG has previously been established by preventing colonization by *S.mutans* and dental caries in animals[4], as well as by preventing both exogenous colonization[12] and recolonization by indigenous *S. mutans*[6] in humans. In this study, the ability of the plant SIgA/G Guy's 13 preparation to prevent recolonization by *S.mutans* was determined alongside the murine IgG1 mAb. Two control groups were included, one that was sham-immunized with saline and another that received a non-specific plant/bovine IgG preparation. The latter control was used to exclude the possibility that a non-specific plant component might co-purify with immunoglobulins and prevent bacterial colonization. It was shown previously that three control murine mAbs have no effect on *S. mutans* recolonization in humans[5,6,12].

The experimental protocol of the *in vivo* study has been described[6]. Briefly, volunteers were chosen who already harboured oral *S.mutans*. They were treated with topical chlorhexidine gluconate for nine days to deplete the oral flora and eliminate *S.mutans*. Thereafter, antibody or control solutions were applied directly to their teeth for three weeks, two applications per week. Samples of dental plaque and saliva were collected at intervals up to four months to monitor the recolonization of *S. mutans*. In every subject, topical chlorhexidine reduced the levels of *S. mutans* to below detectable limits in both plaque and saliva. In saliva (Fig. 4*a*), recolonizing *S.mutans* was initially observed after 21 days in one individual in the group receiving plant/bovine IgG. At 58 days, recolonization of *S. mutans* was found in subjects in both control groups, and levels continued to rise until the end of the experiment at 120 days. In contrast, neither the four subjects treated with the plant SIgA/G preparation nor the four treated with murine Guy's 13 IgG recolonized for the duration of the experiment (Fig. 4*a*). In-plaque (Fig. 4*b*) recolonization started by day 21 in the plant/bovine IgG control group and by day 58 in the saline group and *S. mutans* was present consistently thereafter. Again no recolonization was observed in those subjects who received either the plant SIgA/G or the murine Guy's 13 IgG. Overall, in the saline control group, all subjects had recolonized with *S.mutans* in at least one site by day 88 and in the plant/bovine IgG group by day 58, but there was no significant difference between the rate or levels of recolonization between the two control groups. In contrast, in the groups receiving the SIgA/G or IgG monoclonal antibodies, none of the eight subjects had shown signs of recolonization of *S. mutans* by day 118, neither in saliva nor on the teeth.

The specific nature of this immunotherapeutic approach was tested by monitoring the levels of *actinomyces*, a Gram positive



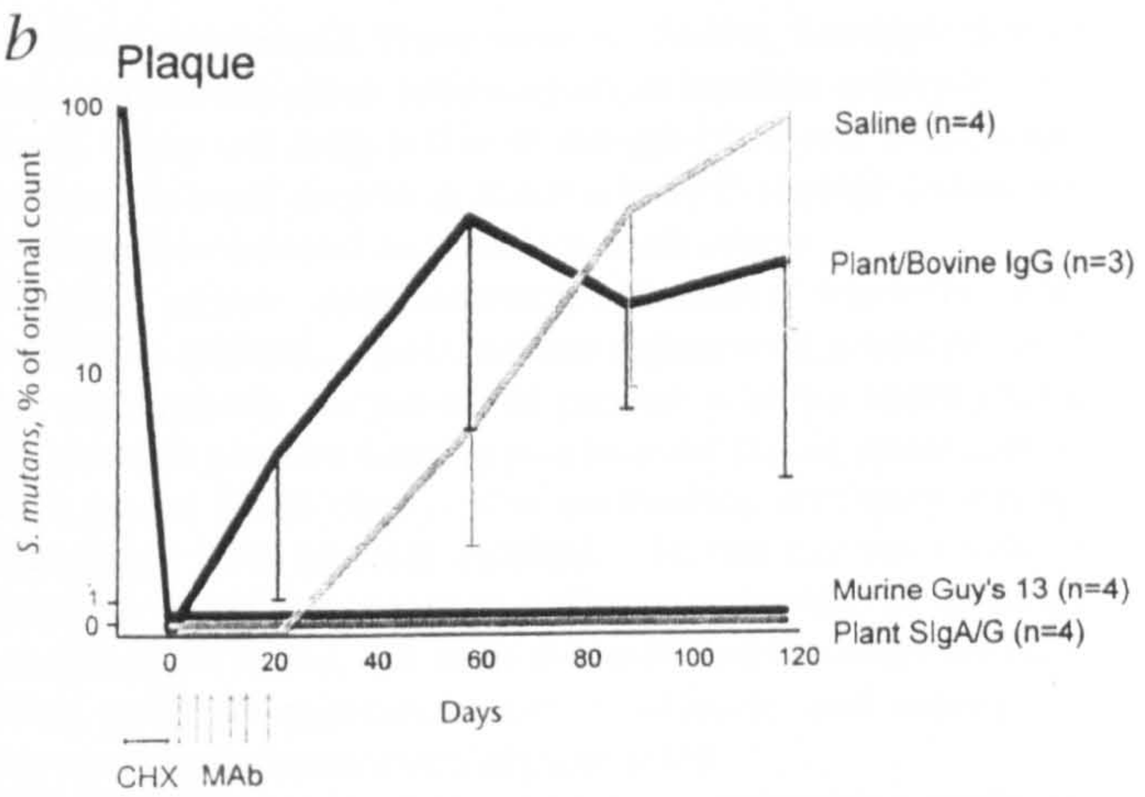


Fig. 4 Recolonization of *S mutans* in the oral cavity in saliva (*a*) and dental plaque (*b*). Results shown are mean levels of *S. mutans* (+sem) expressed as a percentage of the pre-experimental levels set at 100%. Nine days' treatment with chlorhexidine gluconate is indicated, followed by the six applications of immunizing or control solution. The four study groups are indicated and each group comprised four subjects except for the plant/bovine IgG group which had three subjects. Post-chlorhexidine bacteriological samples were taken on days 0, 21, 58, 88 and 118.

commensal bacterial species commonly found in the oral cavity, in subjects receiving plant/bovine IgG or the plant secretory SIgA/G preparation (Fig. 5). As with *S. mutans*, actinomyces fell to undetectable levels following treatment with chlorhexidine, but in contrast to the finding with *S. mutans*, both the non-immunized and immunized groups recolonized within 21 days and the actinomyces were restored to pre-experimental levels by 88 days. This finding supports the specific nature of the action of Guy's 13 mAb, as application of the antibody had no effect on the recolonization of actinomyces or the rate at which this bacterial species was re-established in the oral cavity.

Absence of adverse side effects

A number of clinical parameters were examined at regular intervals to monitor the possibility of any adverse reactions. These included dental and oral mucosal examinations, gingival inflammation[13], dental plaque index[14] and self-reporting by the patients at each visit. Routine hematological screening (blood indices, hemoglobin, erythrocyte sedimentation rate, differential white cell count, serum electrolytes and liver function tests) were performed before the experiment and at day 58. No significant changes were observed in any of the tests in any of the four groups (data not shown). We have also investigated the possibility that the plant SIgA/G preparation might be immunogenic by virtue of either the murine or rabbit components or due to the plant specific glycans associated with the molecule. Pre- and post-immunization serum from all four groups of patients were assayed by ELISA for antibodies that bound to the plant SIgA/G preparation. There were no significant differences between any of the groups in pre-experimental specific IgG, IgA or IgM antibody titres, or binding at 1:50 dilution (IgG) or 1:25 dilution (IgA or IgM) and no significant changes in those subjects that received either the plant SIgA/G or plant/bovine IgG preparations (Fig. 6). This suggests that the plant preparation containing purified antibodies is not immunogenic when given orally.

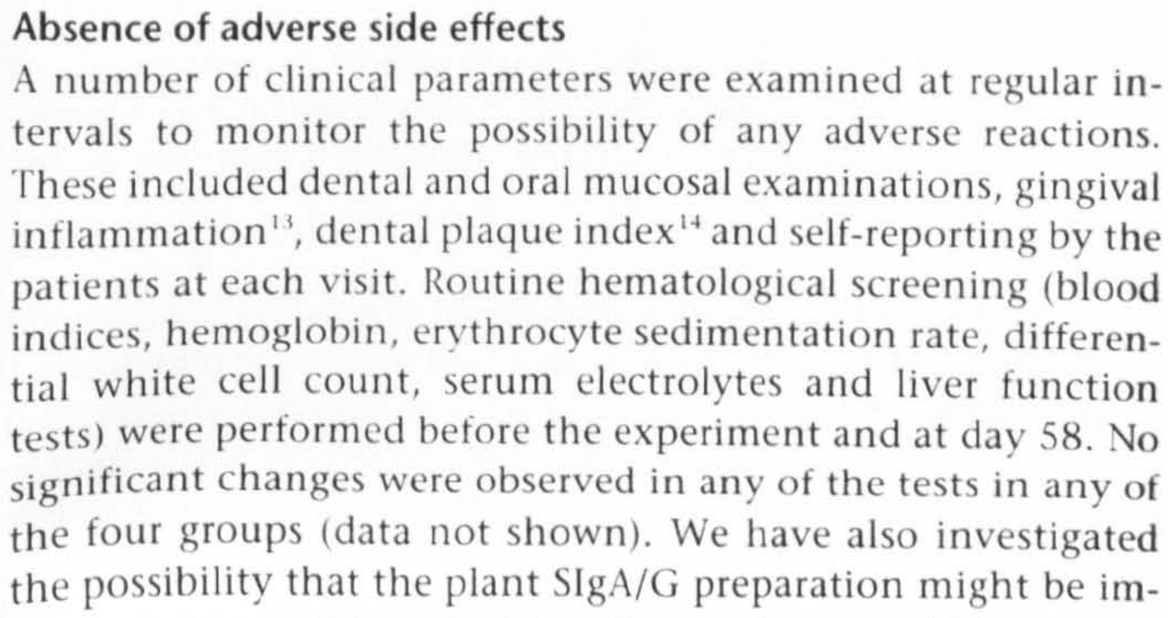


Fig. 5 Re-colonization of *Actinomyces naeslundii* in the oral cavity in dental plaque. Two samples were taken from each patient at each sampling. Results shown, are the mean levels of actinomyces (± sem) expressed as a percentage of the pre-experimental set at 100%. Nine days' treatment with chlorhexidine gluconate is indicated followed by six applications of immunizing or control solutions. Results are shown for plant/bovine IgG control ($n = 3$) and plant SIgA/G groups ($n = 4$) of patients.

Discussion

We have extended our previous findings regarding a monoclonal secretory antibody expressed in transgenic plants. The plant antibodies can readily be extracted and purified resulting in a preparation containing predominantly dimeric antibody species (dIgA/G and SIgA/G), of which the majority is SIgA/G. From a therapeutic point of view, SIgA may provide an advantage over IgG in the mucosal environment[15], although this is not always the case[16]. There are several possible mechanisms of action for protective secretory antibodies at mucosal sites[17,18] and these include neutralization, bacterial aggregation and immune exclusion. In the current model of prevention of *S. mutans* colonisation, there are two main potential benefits to using a monoclonal secretory antibody. These are the increased avidity of secretory antibodies, which enhances bacterial aggregation and immune exclusion, and decreased susceptibility to proteolytic breakdown in the mucosal environment. The dIgA/G present in the plant preparation may also contribute to immune exclusion, but unless it assembles with free human SC in secretions, it is unlikely to survive as long as SIgA/G *in vivo*.

Despite the increased complexity of the plant SIgA/G molecule, the assembly of the antigen binding site is well preserved in plants, resulting in an affinity that is equivalent to that of the original IgG. The plant SIgA/G antibody was shown to have a higher

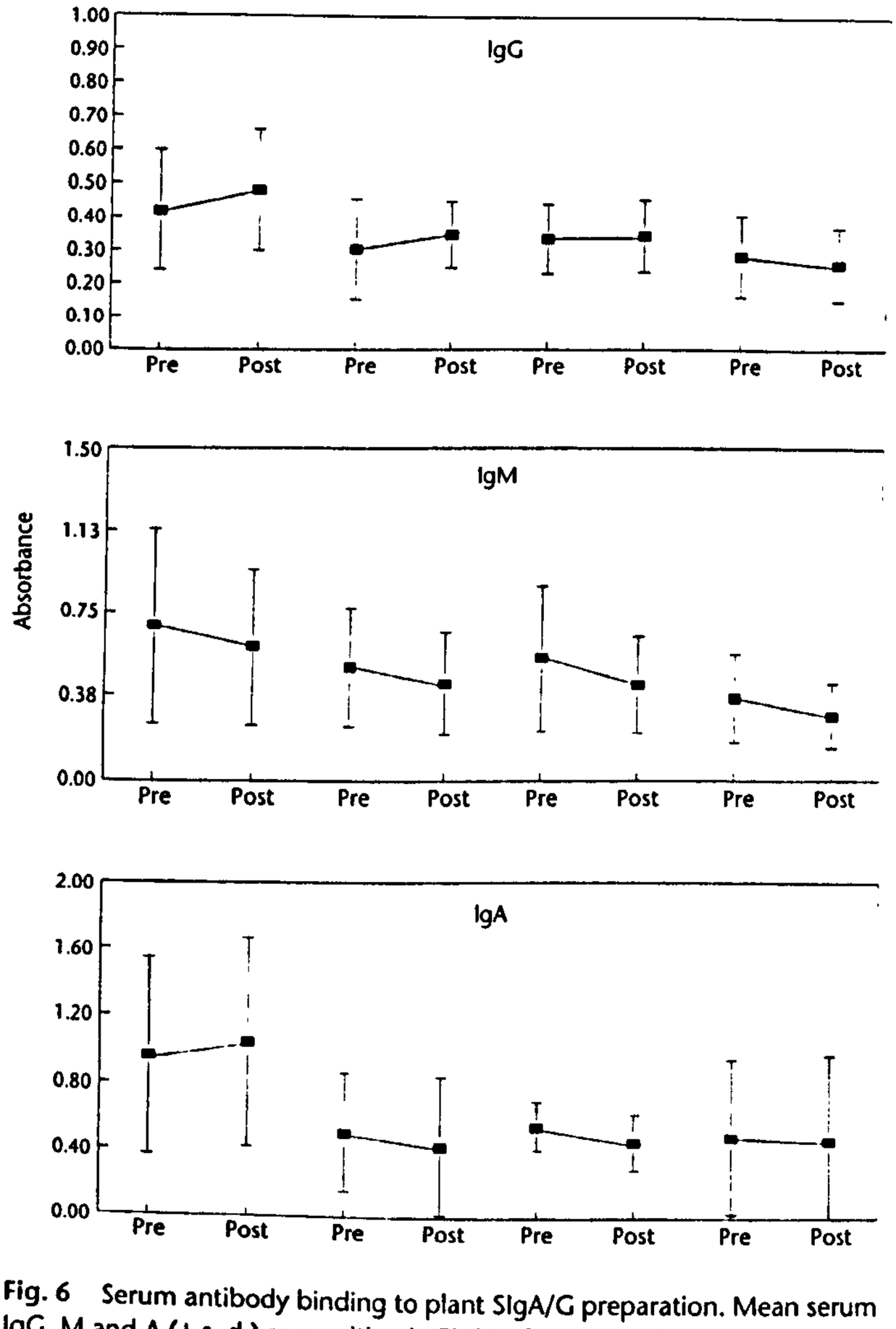


Fig. 6 Serum antibody binding to plant SIgA/G preparation. Mean serum IgG, M and A (± s. d.) recognition in ELISA of the plant SIgA/G preparation is shown for each patient group pre- and post-immunization.

avidity than the IgG due to the dimeric structure of this molecule. We have shown previously that Guy's 13 IgG and $F(ab')_2$ fragment are protective in humans, but the Fab fragment is not[6], suggesting a requirement for at least bivalent antigen binding. Indeed, *in vitro* growth of *S. mutans* with mAb Guy's 13 IgG results in enhanced chaining and aggregation of streptococci[6]. The rationale for the use of the plant SIgA/G antibody is that there is a progressive increase in valency and avidity from the ineffective monomeric Fab, to the protective dimeric $F(ab')_2$ and IgG, to the tetravalent SIgA/G which may correlate with protective efficacy.

A further advantage to using secretory antibodies at mucosal surfaces is the resistance to proteolysis observed with SIgA antibodies[10,11]. Compared with the rest of the gastro-intestinal tract, the mouth does not contain significant amounts of indigenous proteases although potentially there are a number of bacterial proteases. We have shown in a functional *in vivo* assay, that the plant SIgA/G can survive in the oral cavity and be recovered from saliva or teeth for up to three days compared with one day for IgG.

The human trial reported here demonstrates that the plant SIgA/G preparation is as protective and specific as the native mAb Guy's 13 IgG over a period of four months. At this stage we have not optimized the protocol for topical immunotherapy and the advantages of using SIgA/G over IgG may only become evident when optimum minimum therapeutic dose and frequency of application have been determined. There were no clinical, hematological or serological adverse effects following administration of the plant antibody. Given our daily intake of foreign plant and mammalian proteins this is not surprising and it is likely that plant derived antibodies will be safe and acceptable to most people.

Topical passive immunotherapy requires relatively large amounts of antibody. The treatment regime in this trial required 22.5 mg antibody per course of treatment of six applications. This could be purified from approximately 1kg of whole mature plants (about 10–15 plants). The purification efficiency was approximately 69% of total antibody. At the current levels, it should be possible to generate sufficient quantities of antibody for most applications, but there is clearly further scope for optimising antibody expression and purification and subsequent scale up to agricultural levels of production.

This is the first human trial of a monoclonal secretory antibody, as well as of a therapeutic agent produced in transgenic plants (see Tacket *et al.* pg. 605). These findings, along with the combination of scientific, medical and economic benefits of using recombinant plant biotechnology may lead to the development of this approach for other microbial infections affecting mucosal sites, including the gastrointestinal, respiratory and genito-urinary systems of both humans and other animals.

Methods

Antibody purification. Murine IgG Guy's 13 was purified from ascitic fluid by protein G affinity chromatography. Final preparations were in PBS. The fresh plant material was homogenized in 2 volumes of extraction buffer (25 mM Tris pH 7.5, 150 mM NaCl, 10mM EDTA, 1% sodium citrate, 1% PVPP, 0.2% sodium thiosulfate), frozen overnight and thawed. After centrifugation (17,000 *g*, 60 min) the supernatant was filtered through 0.6 µm and 0.2 µm filters and concentrated by diafiltration using a 300-kD molecular weight cutoff MWCO filter (Ultrasette, Pall-Filtron Corporation, Northborough, Massachusetts, USA). Immunoglobulins were precipitated with 50% ammonium sulfate, the precipitate collected by centrifugation (15 min, 17,000 *g*) and re-suspended in PBS. This solution was passed through a protein G affinity column (Poros G, Perseptive Biosystems Inc.) and bound antibody eluted with 3 M potassium thiocyanate. The antibodies were dialyzed against PBS using 100-kD MWCO dialysis tubing (Spectrum), precipitated with 50% ammonium sulfate and resuspended in PBS saline. The resuspended antibodies were dialyzed against phosphate buffered saline using 300-kD MWCO dialysis tubing (Spectrum) and diluted to a concentration of 10 mg/ml before filtration though a 0.2 µm filter. This solution was aliquoted and stored at -20 °C. Endotoxin contamination was measured by the chromogenic limulus amoebocyte lysate assay (Pierce, UK Ltd). Plant SIgA/G antibodies were purified from mature plants.

Non-specific bovine IgG was purified from whole milk by protein G affinity chromotography. This was added to a crude extract from wild-type plants that had been homogenized, filtered and prepared in the same way as the initial transgenic plant preparation. The initial solution contained bovine IgG at a concentration of 16 mg IgG/kg fresh plant material. The IgG was then recovered similarly to the Guy's 13 SIgA/G. The final preparation was at 10 mg/ml in PBS and sterilized by filtration through a 0.2 µm filter. No specific binding to either *S. mutans* cells or purified SA I/II could be detected by ELISA (result not shown). Aliquots were stored at -20 °C.

SDS-PAGE and western blot. For SDS-PAGE, the purified SIgA/G preparation samples were boiled for three minutes in 75mM Tris-HCl (pH 6.8), 2% SDS, separated on a 7.5% acrylamide gel and proteins were stained with Coomasie Blue. For western blot, unpurified plant extracts were prepared in 1M Tris-HCl (pH 8.45), 8% SDS, 8 M urea, 20% glycerol and analyzed on a 5–18% Tris-Tricine gradient gel. The gel was blotted onto nitrocellulose and incubated (2 h, 37 °C) in PBS with 0.05% Tween-20 and 1% non-fat dry milk. Detection of individual lanes was with horseradish peroxidase conjugated goat anti-mouse kappa chain, goat anti-mouse alpha chain, goat anti-mouse gamma chain antiserum (Southern Biotechnology Associates, Inc.,

USA), rabbit anti-human J chain antiserum (donated by B. Corthesy) and sheep anti-rabbit SC antiserum (donated by B. Underdown) followed by horseradish peroxidase conjugated goat anti-rabbit IgG (H+L) or anti-sheep IgG (H+L) (Southern Biotechnology Associates, Inc., USA) respectively, as indicated. Bound antibodies were detected by chemiluminescence (ECL Amersham).

Antibody affinity measurement. Antibody affinities were measured using the Biacore X system (Biacore AB, Sweden). Approximately 700 resonance units of streptococcal antigen (SA) I/II were immobilised on a CM5 sensor chip (Biacore AB, Sweden) in 10 mM sodium acetate buffer pH 4. The binding kinetics of the murine IgG and plant SIgA/G Guy's 13 were measured at concentrations of 50, 62.5, 75, 87.5 and 100 nM at a flow rate of 10 ml/min. After each binding measurement, the surface was regenerated with 100 mM HCl.

Competition ELISA for estimation of antibody avidity. This assay was performed as described[8]. Briefly, Immulon IV 96-well microplates (Dynatech), coated with SA I/II (2 μg/ml), were incubated with twofold serial dilutions of SA I/II (1–100 nM) together with SIgA/G or IgG Guy's 13 (at 4.50 nM antibody combining site). Detection of antibody binding to the solid-phase antigen was performed by incubation with a biotinylated rabbit anti-mouse IgA, G and M conjugate (Boehringer Mannheim, UK Ltd), followed by streptavidin-peroxidase conjugate (Boehringer Mannheim, UK Ltd.,) and tetramethylbenzydine dihydrochloride (TMB; Sigma UK Ltd.).

Detection of antibody survival *in vivo*. A single application of murine mAb Guy's 13 IgG, plant SIgA/G or BSA in PBS was administered to each of two volunteers. Thirty minutes following an oral prophylaxis with pumice and saline, 5 μl of each solution (10 mg/ml concentration) was applied to every tooth surface. Prefabricated impression trays were inserted and kept in place for five minutes and the subjects were requested not to eat, drink or rinse for 30 minutes. Otherwise no restrictions were placed on their diet or oral hygiene practice. At intervals of 0.5, 1.5, 6, 24, 48, 72 and 144 hours, samples of saliva were collected in addition to samples of plaque from four teeth—the latter were collected using cotton wool moistened with sterile saline and pumice—and immediately stored at -70 °C. Collection fluids were separated by centrifugation and assayed by an antigen specific ELISA. Microtitre plates were coated with purified SA I/II (2 μg/ml) in TBS (150 mm NaCl, 20 mm Tris-HCl pH 8) and blocked with 5% non-fat dry milk in TBS at 37 °C for two hours. Saliva was diluted 1:32 with sterile PBS. Oral samples were added in triplicate, in serial 2-fold dilutions to the microtitre plate; incubation was at 4 °C overnight. After washing with TBS with 0.05% Tween 20, either a goat anti-mouse κ chain antiserum or a sheep anti-serum to rabbit secretory component was added for two hours at 37 °C. After further washing, an appropriate second layer alkaline phosphatase conjugated antibody was applied for two hours at 37 °C. Antibody binding was detected with disodium p-nitrophenyl phosphate (Sigma UK) and absorbance recorded at 405 nm.

Microbiological sampling of human volunteers. Dental plaque samples were collected from the cervical and fissural surfaces of all central incisors and first molars as described[6]. Whole saliva was collected directly into sterile tubes cooled to 4 °C. Samples were plated in serial dilutions onto TYC agar (Lab M, UK) supplemented with 20% sucrose and 0.1 U/ml bacitracin TYCSB plates and identified by their characteristic morphology and fermentation of specific sugars[19]. Total anaerobic counts were determined on the blood agar plates. For determination of actinomyces, samples were plated onto CFAT medium[20] and incubated in 90% air/10% CO_2 for three days. *A. naeslundii* was identified by its characteristic appearance and positive Gram stain. Pre-experimental levels of *S. mutans* and *A. naeslundii* were determined from three samples taken over an eight day period.

Local passive immunization. Volunteers used 10 ml 0.2% chlorhexidine gluconate mouthwash (Corsodyl, Smithkline Beecham, UK) twice a day and had 1% chlorhexidine gel applied daily in stock gel application trays (Nupro, Johnson and Johnson, UK) for nine days. On the following day, 30 minutes before the first application of mAb or control solution, teeth were professionally cleaned and an aliquot of 5 μl was then applied to all surfaces of each tooth using a Gilson Pipetman. Custom made silicone impression trays (Optosil and Xantopren, Bayer Dental, UK) were inserte for five minutes to prevent washing away or dilution by saliv Subsequent treatments were performed without prior cleaning of t teeth. In all, six applications were carried out on days 1, 4, 8, 11, 15 ar 18.

Serum antibody reactivity to plant SIgA/G preparation. Serum sampl were collected before the experiment and one month after the last applic tion of topical test solution. For IgG the serum was diluted 1/50 in TBS ar for IgA and IgM the dilution was 1/25. These were applied in triplicate ELISA plates that had been coated with the SIgA/G preparation in TBS (at previously determined optimal concentration of 2 μg/ml) and blocked wi 5% non-fat dry milk in TBS. Incubation was at 37 °C for two hours. Aft washing with TBS with 0.05% Tween 20, bound immunoglobulins were l belled using alkaline phosphatase conjugated goat anti-sera to human IgG M or A (Sigma UK) for two hours at 37 °C. After further washing, detectio was with disodium p-nitrophenyl phosphate (Sigma UK). Absorbance wa determined at 405 nm.

Acknowledgements

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1. Arntzen, C.J. High-tech herbal medicine: plant-based vaccines [news]. *Nature Biotec* **15**, 221–222 (1997).
2. Hiatt, A.C., Cafferkey, R. & Bowdish, K. Production of antibodies in transgenic plant *Nature* **342**, 76–78 (1989).
3. Ma, J.K-C. *et al.* Generation and assembly of secretory antibodies in plants. *Scien* **268**, 716–719 (1995).
4. Lehner, T., Caldwell, J. & Smith, R. Local passive immunization by monoclonal ant bodies against streptococcal antigen I/II in the prevention of dental caries. *Infec Immun.* **50**, 796–799 (1985).
5. Ma, J.K., Smith, R. & Lehner, T. Use of monoclonal antibodies in local passive immu nization to prevent colonization of human teeth by *Streptococcus mutans*. *Infect. Immun.* **55**, 1274–1278 (1987).
6. Ma, J.K-C., Hunjan, M., Smith, R., Kelly, C.& Lehner, T.An investigation into th mechanism of protection by local passive immunisation with monoclonal antibodie against *Streptococcus mutans*. *Infect. Immun.* **58**, 3407–3414 (1990).
7. Ma, J.K-C., Lehner, T., Stabila, P., Fux, C.I. & Hiatt, A. Assembly of monoclonal ant bodies with IgG1 and IgA heavy chain domains in transgenic tobacco plants. *Eur. Immunol.* **24**, 131–138 (1994).
8. Steward, M.W. & Chargelegue, D. Overview: Measurement and biological signif cance of antibody affinity and reactivity. In D.M. Weir, L.A. Herzenberg and C.C Blackwell (eds.), *Handbook of experimental immunology*, Blackwell Scientific, Oxfor 1997, pp. 38.1.
9. Underdown, B.J. & Dorrington, K.J. Studies on the structural and conformational bas for the relative resistance of serum and secretory immunoglobulin A to proteolysis. *Immunol.* **112**, 949–959 (1974).
10. Mestecky, J. & McGhee, J.R. Immunoglobulin A (IgA): molecular and cellular intera tions involved in IgA biosynthesis and immune response. *Adv. Immunol.* **40**, 153–24 (1987)
11. Corthesy, B. Recombinant secretory IgA for immune intervention against mucosa pathogens. *Biochem. Soc. Trans.* **25**, 471–475 (1997)
12. Ma, J.K., Hunjan, M., Smith, R. & Lehner, T. Specificity of monoclonal antibodies i local passive immunization against *Streptococcus mutans*. *Clin. Exper. Immunol.* **7** 331–337 (1989)
13. Loe, H. & Silness, J. Periodontal disease in pregnancy. I. Prevalence and severity. *Act Odontol. Scand.* **21**, 532–551 (1963)
14. Silness, J. & Loe, H. Periodontal disease in pregnancy. II. Correlation between oral hy giene and periodontal condition. *Acta Odontol. Scand.* **22**, 121—135 (1964)
15. Hajishengallis, G., Nikolova, E. & Russell, M.W. Inhibition of Streptococcus mutan adherence to saliva-coated hydroxyapatite by human secretory immunoglobulin (S-IgA) antibodies to cell surface protein antigen I/II: reversal by IgA1 protease cleav age. *Infect. Immun.* **60**, 5057–5064 (1992)
16. Mazanec, M.B., Lamm, M.E., Lyn, D., Portner, A. & Nedrud, J.G. Comparison of Ig versus IgG monoclonal antibodies for passive immunization of the murine respirato tract. *Vir. Res.* **23**, 1–12 (1992)
17. Childers, N.K., Bruce, M.G. & McGhee, J.R. Molecular mechanisms of immunoglob lin A defense. *Ann. Rev. Microbiol.* **43**, 503–536 (1989)
18. Mazanec, M.B., Nedrud, J.G., Kaetzel, C.S. & Lamm, M.E. A three-tiered view of th role of IgA in mucosal defense. *Immunol. Today* **14**, 430–435 (1993)
19. Beighton, D., Russell, R.R. & Whiley, R.A. A simple biochemical scheme for the diffe entiation of *Streptococcus mutans* and *Streptococcus sobrinus*. *Caries Res.* **25**, 174–17 (1991)
20. Zylber, L.J. & Jordan, H.V. Development of a selective medium for detection and en meration of *Actinomyces viscosus* and *Actinomyces naeslundii* in dental plaque. *J. Cli Microbiol.* **15**, 253–259 (1982)

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SCIENCE

Generation and Assembly of Secretory Antibodies in Plants

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Four transgenic *Nicotiana tabacum* plants were generated that expressed a murine monoclonal antibody kappa chain, a hybrid immunoglobulin A-G heavy chain, a murine joining chain, and a rabbit secretory component, respectively. Successive sexual crosses between these plants and filial recombinants resulted in plants that expressed all four protein chains simultaneously. These chains were assembled into a functional, high molecular weight secretory immunoglobulin that recognized the native streptococcal antigen I/II cell surface adhesion molecule. In plants, single cells are able to assemble secretory antibodies, whereas two different cell types are required in mammals. Transgenic plants may be suitable for large-scale production of recombinant secretory immunoglobulin A for passive mucosal immunotherapy. Plant cells also possess the requisite mechanisms for assembly and expression of other complex recombinant protein molecules.

Secretory immunoglobulin A (SIgA) is the most abundant form of immunoglobulin (Ig) in mucosal secretions, where it forms part of the first line of defense against infectious agents. The molecule exists mainly in the 11S dimeric form, in which two monomeric IgA antibody units are associated with the small polypeptide joining (J) chain and with a fourth polypeptide, secretory component (SC). The ability to produce monoclonal SIgA would be of substantial value, but the synthesis is complicated because it requires plasma cells secreting dimeric IgA (dIgA) as well as epithelial cells expressing the polymeric Ig receptor (pIgR). Normally, pIgR on the epithelial basolateral surface binds dIgA, initiating a process of endocytosis, transcytosis, phosphorylation, proteolysis, and ultimate release of the SIgA complex at the apical surface into the secretion (1). Here, we focused on the ability of transgenic plants to assemble secretory antibodies.

Genes encoding the heavy and light chains of a murine antibody (Guy's 13), a murine J chain, and a rabbit SC were introduced into separate transgenic tobacco plants. Guy's 13 is a murine IgG1 monoclonal antibody (mAb) that recognizes the streptococcal antigen (SA) I/II cell surface adhesion molecule of *Streptococcus mutans* and *S. sobrinus* (2). Transgenic full-length Guy's 13 has been generated in *N. tabacum* plants and was found to be correctly assembled (3). Modification of the heavy chain by replacement of its Cγ3 domain with Cα2 and Cα3 domains from an IgA-secreting hybridoma (MOPC 315) did not affect the assembly or function of the antibody (IgA-G) produced in transgenic plants (3). Protein immunoblot analysis (4) of the IgA-G plant extract with antiserum to the κ light chain under nonreducing conditions showed a band of ~210 kD, which is consistent with the presence of the extra constant region domains in the IgA-G antibody construct as compared with the original IgG1 antibody (Fig. 1A, lanes 1 and 3). A number of smaller proteolytic fragments were also detected, which is consistent with previous findings (3). A mouse J chain construct that consisted of coding-length complementary DNA (cDNA) was amplified with synthetic oligonucleotide primers corresponding to the NH_2-terminal MKTHLL and the COOH-terminal SCYPD sequences of the mouse J chain (5). The SC construct used in this study consisted of coding-length cDNA amplified with synthetic oligonucleotide primers corresponding to the NH_2-terminal MALFLL sequence and the AVQSAE sequence near the COOH-terminus of rabbit pIgR (6). Transgenic plants were then regenerated (7).

The plants that expressed the J chain were crossed with those expressing IgA-G. The progeny showed a second major Ig band at ~400 kD, approximately twice the relative molecular mass of the IgA-G molecule (Fig. 1A, lane 4), which suggested that a dimeric antibody (dIgA-G) had been assembled. Mature plants that expressed dIgA-G were crossed with a homozygous plant that expressed SC. The progeny plants (SIgA-G) included those that produced a higher mo-

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lecular mass band of ~470 kD in protein immunoblot analysis under nonreducing conditions (Fig. 1A, lane 5); such a molecular size is consistent with that expected for a secretory Ig. Detection with antiserum to SC confirmed that this high molecular mass protein contained SC (Fig. 1A, lane 7). The plant extracts also contained the 400-kD band (dIgA-G) and the 210-kD band (IgA-G), but these were detected only by antiserum to the κ light chain and not by antiserum to SC. In the transgenic plant that secreted SC alone, no high molecular mass proteins were detected in protein immunoblotting under nonreducing conditions (Fig. 1A, lane 9), and hence there was no evidence that SC assembled with endogenous plant proteins or formed multimers.

Further protein immunoblot analysis under reducing conditions demonstrated that extracts from the plants that expressed antibodies (IgA-G, dIgA-G, and SIgA-G), but not those that expressed the J chain or SC, contained identical antibody heavy and light chains (Fig. 1B, upper and middle panels). Only the SC and SIgA-G plants expressed proteins that were recognized by antiserum to SC (lower panel). The dissociation of SC from Ig heavy chains only under reducing conditions suggests that the SC chain was at least partially covalently linked in the assembled SIgA-G molecule. The molecular mass of the major SC band under reducing conditions is ~50 kD, which is lower than expected (66.5 kD). This is probably a result of proteolysis, which may occur in the intact plant or during sample preparation. SC bound to dimeric IgA is often found proteolyzed to smaller but biologically active forms in vivo (*8*). However, in the protein immunoblot analysis under nonreducing conditions, the molecular mass difference between dIgA-G and SIgA-G was ~70 kD, as expected (Fig. 1A). No cross-reacting proteins were detected in extracts from the wild-type control plant.

In mammals, the assembly of SC with antibody requires the presence of the J chain (*9*); we next investigated whether this was also the case in plants. Plants expressing monomeric IgA-G were crossed with SC-expressing plants. In the progeny, only the 210-kD monomeric form of the antibody was recognized by antiserum to the κ light chain (Fig. 2A, lanes 1 and 2); antiserum to SC recognized free SC but did not recognize proteins associated with Ig (Fig. 2B). This result was confirmed in all 10 plants examined, whereas all 10 plants that coexpressed the J chain, the antibody chains, and SC assembled the 470-kD SIgA-G molecule. This finding confirms the requirement of the J chain for SC association with Ig and suggests that the nature of the association in plants is similar to that in mammals.

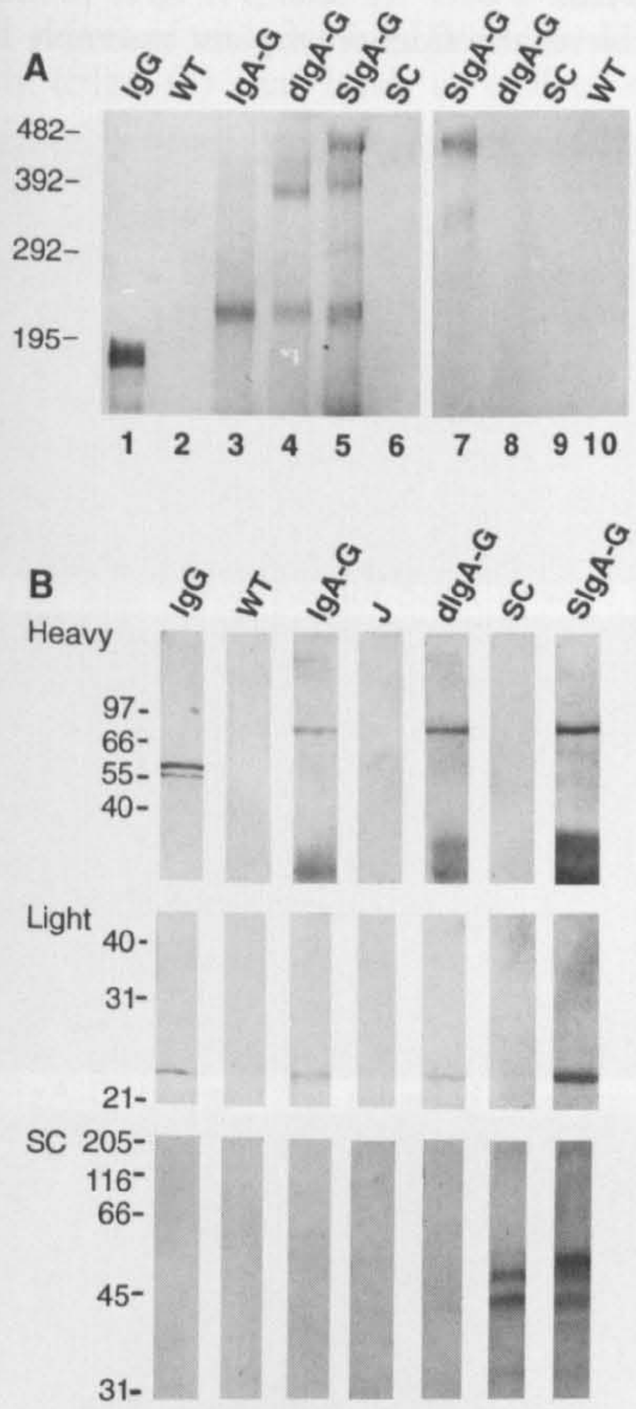

Fig. 1. (**A**) Protein immunoblot of plant extracts prepared under nonreducing conditions, detected with antisera to the mouse κ light chain (lanes 1 to 6) or to rabbit SC (*24*) (lanes 7 to 10). Samples were prepared (*4*) and separated on 4% SDS–polyacrylamide electrophoresis gel (SDS-PAGE). (**B**) Protein immunoblot of plant extracts prepared under reducing conditions. Samples were prepared as in (*4*), but with the addition of 5% β-mercaptoethanol. SDS-PAGE in 10% acrylamide was performed and the gels were blotted as before. Detection was with antisera to the mouse γ1 heavy chain (upper panel), the mouse κ light chain (middle panel), or rabbit SC (lower panel), followed by the appropriate second-layer alkaline phosphatase–conjugated antibody. IgG, Guy's 13 mAb (*2*) prepared in hybridoma cell culture supernatant; WT, nontransformed wild-type plant; IgA-G, transgenic plant expressing modified heavy and light chain genes of Guy's 13; dIgA-G, transgenic plant expressing modified heavy and light chain genes of Guy's 13 and the J chain; SIgA-G, transgenic plant expressing modified heavy and light chain genes of Guy's 13, the J chain, and SC; SC, transgenic plant expressing SC; and J, transgenic plant expressing the J chain. Molecular masses are indicated in kilodaltons.

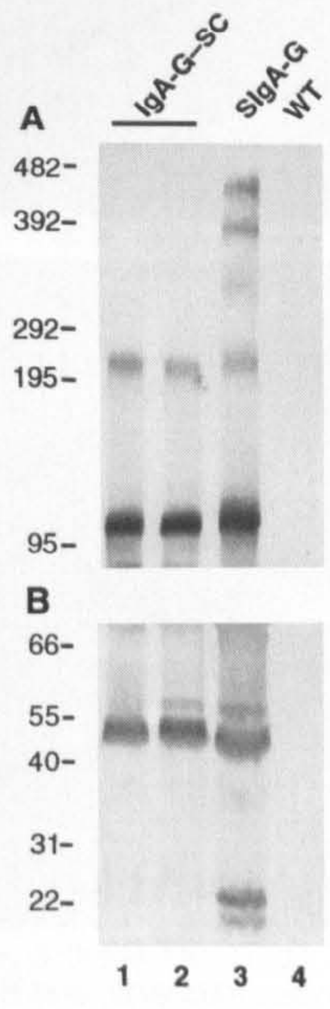

Fig. 2. Coexpression of IgA-G with SC. Protein immunoblotting of transgenic plant extract (*4*) was performed (**A**) under nonreducing conditions on 4% SDS-PAGE and detected with goat antiserum to the κ light chain, followed by alkaline phosphatase–labeled rabbit antiserum to goat IgG, and (**B**) under reducing conditions on 10% SDS-PAGE and detected with sheep antiserum to SC, followed by alkaline phosphatase–labeled donkey antiserum to sheep IgG. IgA-G–SC, transgenic plant expressing modified heavy and light chain genes of Guy's 13 and SC.

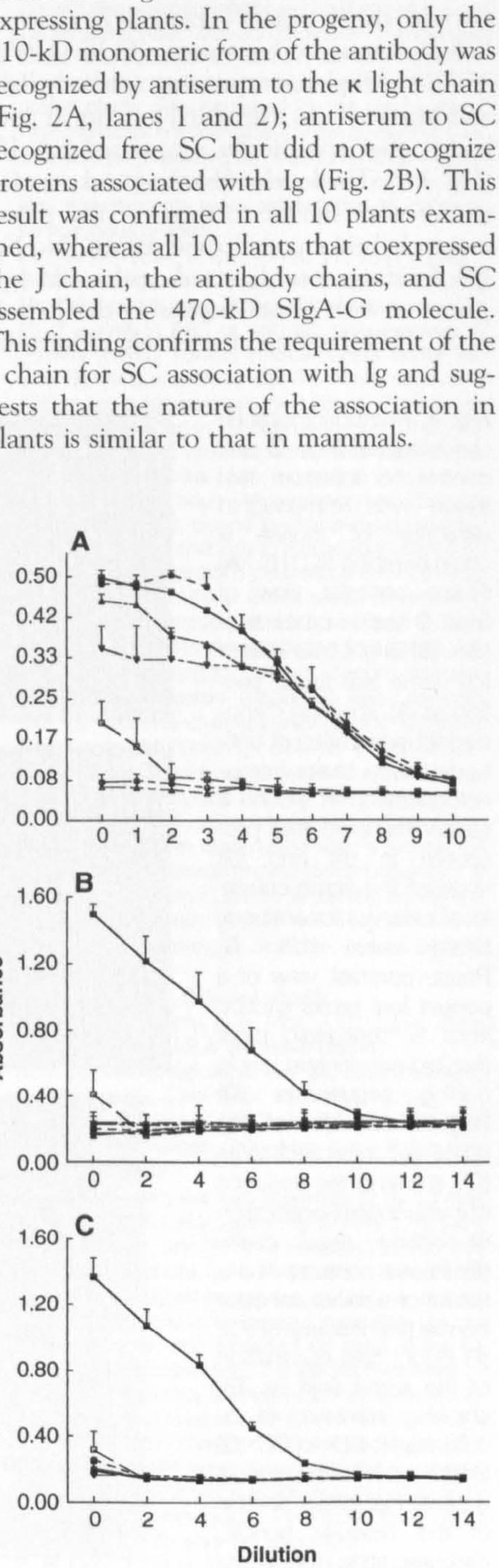

Fig. 3. Functional antibody expression in transgenic *N. tabacum*, as measured by absorbance at 405 nm (A_{405}). (**A**) Plant extract binding to purified SA I/II, detected with HRP-labeled antiserum to the κ light chain. (**B**) Plant extract binding to purified SA I/II, detected with sheep antiserum to SC followed by alkaline phosphatase–labeled donkey antiserum to sheep Ig. (**C**) Plant extract binding to streptococcal cells, detected with sheep antiserum to SC followed by alkaline phosphatase–labeled donkey antiserum to sheep Ig. Guy's 13 hybridoma cell culture supernatant (IgG) was used as a positive control. The initial concentration of each antibody solution was 5 μg/ml. Dilution numbers represent serial double dilutions. Results are mean ± SD of three separate triplicate experiments. ■, SIgA-G; ●, dIgA-G; ▲, IgA-G; □, SC; ○, J; △, WT; and ▼, Guy's 13.

Functional antibody studies were carried out with the five plant constructs by enzyme-linked immunosorbent assay (ELISA) (Fig. 3) (*10*). All plants expressing antibody light and heavy chains assembled functional antibodies that specifically recognized SA I/II (Fig. 3A). The levels of binding and titration curves were similar to those of the native mouse hybridoma cell supernatant. No SA I/II binding was detected with wild-type plants or with plants expressing the J chain or SC. The binding of antibody to immobilized purified SA or native antigen on the bacterial cell surface was also detected with antiserum to SC (Fig. 3, B and C). In these assays, only the SIgA-G plant antibody binding was detected and not the functional antibodies in the IgA-G or dIgA-G plants. These results confirm that SC was assembled with antibody in the SIgA-G plant but did not interfere with antigen recognition or binding.

The assembly of functional Ig molecules in plants is very efficient (*11*). Initial estimates for the plants expressing SIgA-G suggest that approximately 50% of the SC is associated with dimeric IgA-G in the plant extracts (*12*). Preliminary results indicate that the SIgA-G yield from fully expanded leaf lamina is 200 to 500 μg per gram of fresh weight material. This yield is considerably greater than that determined for monomeric IgA-G and is consistent with the suggestion that SIgA-G might be more resistant to proteolysis.

Here, the fidelity of plant assembly has

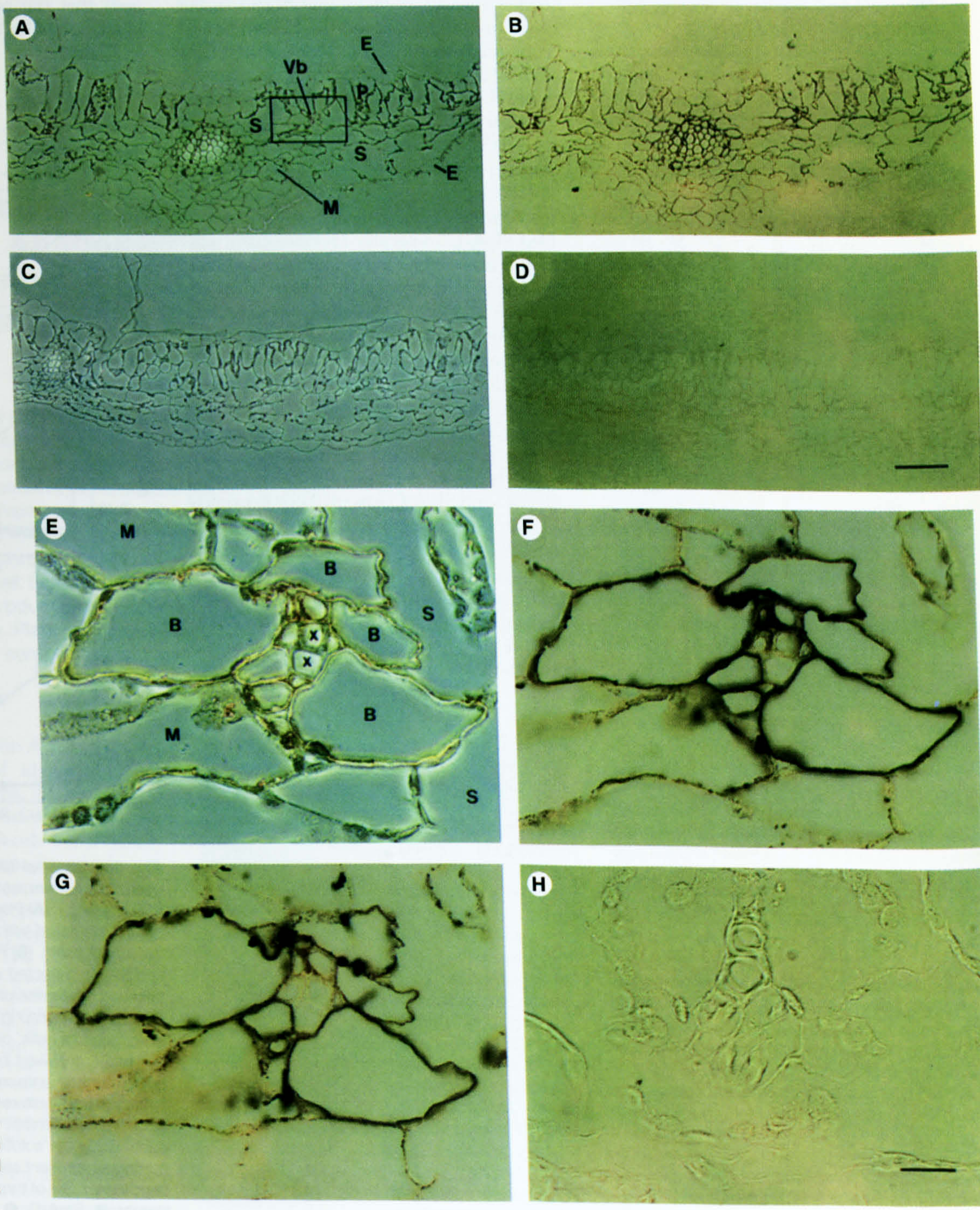

Fig. 4. Photomicrographs of transgenic SIgA-G and control *N. tabacum* leaf tissue with immunogold detection of murine α chain or rabbit SC (*16*). (**A**) Phase contrast view of SIgA-G leaf in cross section. (**B**) Bright field view of the same leaf cross section as (A), showing immunogold localization of antibody heavy chain. Immunolocalization of SC on a section serial to that presented in (A) and (B) showed the same cellular localization as for antibody heavy chain (*12*). (**C**) Phase contrast view of a control leaf cross section from a transgenic plant that did not contain any Ig coding sequences. (**D**) Bright field view of the same leaf cross section as (C), showing the absence of immunogold localization of antibody heavy chain. (**E**) Phase contrast illumination of a minor vascular bundle [framed area of (A)]. (**F**) Bright field illumination of the same field as (E), showing immunogold localization (black) of SC. (**G**) Bright field illumination of a serial leaf cross section of the vascular bundle, demonstrating the same immunogold localization (black) of antibody heavy chain as that shown in (F). (**H**) Bright field illumination of a control leaf vascular bundle in cross section, showing absence of immunogold localization of antibody heavy chain. A control leaf section was also incubated with the gold-labeled secondary antibodies alone, and no binding was observed (*12*). M, spongy mesophyll cells; E, epidermal cells; S, intercellular spaces; P, palisade parenchyma cells; Vb, vascular bundles; X, xylem tracheids; and B, bundle sheath cells. Scale bars: 100 μm (A through D), 10 μm (E through H).

been extended to include dimerization of monomeric antibody by the J chain. Coexpression of recombinant IgA with the J chain through the use of baculovirus in insect cells has been reported (*13*); however, only a small proportion of the expressed antibody was dimerized, and most remained in a monomeric form. By contrast, in plants the dimeric antibody population represents a major proportion (~57%) of the total antibody (Fig. 1A, lane 4). This is also the first report of an assembled secretory antibody (SIgA-G) that binds as well to the corresponding antigen as does the parent mAb and constitutes a major proportion of the total assembled antibody (~45%; Fig. 1A, lane 5). Protein immunoblot analysis potentially underestimates the total extent of assembly of SIgA-G because it only detects antibody that is covalently linked to SC, whereas SIgA can occur in vivo as a mixture of covalently and noncovalently linked molecules (*14*).

The four transgenes for SIgA-G were introduced into plants with the identical pMON530 expression cassette, native leader sequences, and a promoter sequence derived from the 35S transcript of the cauliflower mosaic virus, which directs expression of transgenes in a variety of cell types of most plant organs (*15*). The use of the same promoter for all four transgenes maximized the likelihood of coincidental expression in a common plant cell. Microscopic observation of SIgA-G plants (*16*) revealed that many cell types of the leaves contained SIgA-G components (Fig. 4, A and B). The predominant accumulation of these proteins was in the highly vacuolated cells of the mesophyll, particularly in bundle sheath cells; the cytoplasmic band surrounding the large central vacuole was strongly labeled (Fig. 4, F and G). At the level of light microscopy, it is not possible to distinguish between antigens that are cytoplasmic and those that are contained in the luminal apoplastic space between the cell wall and the plasmalemma, but it is evident that the recombinant antibody components do not penetrate the cell wall.

Restriction of the largest SIgA-G components, SC and heavy chain, within the confines of the protoplastic or apoplastic compartments of individual cells would constrain the assembly of SIg to single cells. In contrast, two cell types are required to produce SIgA in mammals. In the plant system, a mature SC devoid of signals for membrane integration, transcytosis, or subsequent proteolysis can thus be assembled with a hybrid Ig containing α domains within the secretory pathway of the cell. Assembly of monomeric antibody is known to require the targeting of both light and heavy chains to the endoplasmic reticulum (ER) (*17*). Thus SIgA-G assembly might occur at two sites: either in the ER, after dimerization with the J chain, or in the extracellular apoplasm, where the secreted antibody is accumulated.

The inherent functions of IgG-constant regions, that is, protein A binding, complement fixation, and the ability to bind to specific cell surface receptors (Fc receptors), may be retained in a dimeric Ig that is capable of binding SC. These additional properties of SIgA-G may enhance the function of the complex in passive immunotherapy, although under some circumstances these biological properties might be undesirable. In principle it should not be difficult to produce a SIgA-G antibody that lacks the Cγ2 domain in these cases.

The development of plants capable of generating functional SIgA may have significant implications for passive immunotherapy. Previously, SIgA has been generated only with difficulty, by in vitro conjugation of SC with dimeric IgA (*18*) or by the insertion of subcutaneous "backpack" tumors of hybridoma cells secreting monoclonal IgA (*19*). The plants express SIgA in large amounts, and the production can be scaled up to agricultural proportions. This method offers an economic means of producing large quantities of mAbs that could be applied to mucosal surfaces to prevent infection, as has been demonstrated in passive immunotherapy against streptococci (*20*). Multivalent antibodies might be more protective than IgG at mucosal surfaces (*21*), and SC may also have postsecretory functions in stabilizing the polymeric antibody against proteolysis (*22*). The principle of sexual crossing of transgenic plants to accumulate recombinant subunits can readily be applied to the assembly of a variety of Ig as well as other complex protein molecules.

REFERENCES AND NOTES

1. K. E. Mostov, *Annu. Rev. Immunol.* **12**, 63 (1994).
2. R. Smith and T. Lehner, *Oral Microbiol. Immunol.* **4**, 153 (1989).
3. J. K.-C. Ma, T. Lehner, P. Stabila, C. I. Fux, A. Hiatt, *Eur. J. Immunol.* **24**, 131 (1994).
4. Leaf segments were homogenized in tris-buffered saline (TBS) [150 mM NaCl and 20 mM tris-HCl (pH 8)] with leupeptin (10 μg/ml) (Calbiochem). The extracts were boiled for 3 min in 75 mM tris-HCl (pH 6.8) and 2% SDS under nonreducing conditions, and SDS–polyacrylamide gel electrophoresis (PAGE) in 4 or 10% acrylamide was performed. The gels were blotted onto nitrocellulose. The blots were incubated for 2 hours in TBS with 0.05% Tween 20 (Merck Ltd., Leicester, UK) and 1% nonfat dry milk, followed by the appropriate antiserum, and were incubated for 2 hours at 37°C. After washing, the appropriate second-layer alkaline phosphatase–conjugated antibodies were applied for 2 hours at 37°C. Antibody binding was detected by incubation with nitroblue tetrazolium (300 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (150 mg/ml).
5. L. Matsuuchi, G. M. Cann, M. E. Koshland, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 456 (1986). Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; H, His; K, Lys; L, Leu; M, Met; P, Pro; Q, Gln; S, Ser; T, Thr; V, Val; and Y, Tyr.
6. K. E. Mostov, M. Friedlander, G. Blobel, *Nature* **308**, 37 (1984).
7. DNA fragments were ligated into pMON 530 (*23*), a constitutive plant expression vector that includes the cauliflower mosaic virus 35S promoter. Tobacco leaf tissue was transformed with the use of an agrobacterium containing the recombinant plasmid. Regenerated plants were screened for the production of RNA transcript encoding the J chain by reverse transcriptase polymerase chain reaction and for the production of SC by protein immunoblot analysis. Positive transformants were self-fertilized to generate homozygous progeny.
8. D. J. Ahnen *et al.*, *J. Clin. Invest.* **77**, 1841 (1986).
9. P. Brandtzaeg and H. Prydz, *Nature* **311**, 71 (1984).
10. Microtiter plates were coated either with purified SA I/II (2 μg/ml) in TBS or with log phase growth *S. mutans* (NCTC 10449) in bicarbonate buffer (pH 9.8). Blocking was done with 5% nonfat dry milk in TBS at room temperature for 2 hours. Plant leaves were homogenized in TBS with leupeptin (10 μg/ml). The supernatants were added in serial twofold dilutions to the microtiter plate; incubation was at room temperature for 2 hours. After washing with TBS with 0.05% Tween 20, bound Ig chains were detected either with a goat antibody to mouse light chain conjugated with horseradish peroxidase (HRP) (Nordic Pharmaceuticals, UK) or with a sheep antiserum to SC, followed by donkey antibody to sheep Ig, conjugated with alkaline phosphatase. Conjugated antibodies were applied for 2 hours at room temperature. HRP-conjugated antibodies were detected with 2,2′-azino-di-(3-ethyl-benzthiazoline sulfonate) (Boehringer Mannheim); alkaline phosphatase–conjugated antibodies were detected with disodium *p*-nitrophenylphosphate (Sigma, UK). The concentrations of the antibody solutions were initially determined by ELISA in comparison with a mouse IgA mAb (TEPC 21) used at known concentrations (*3*). In the antigen-binding ELISAs, the starting concentration of each antibody solution was 5 μg/ml.
11. A. Hiatt, R. Cafferkey, K. Bowdish, *Nature* **342**, 76 (1989).
12. J. K.-C. Ma *et al.*, data not shown.
13. L. Carayannopoulos, E. E. Max, J. D. Capra, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 8348 (1994).
14. R. D. Schneiderman *et al.*, *ibid.* **86**, 7561 (1989); K. L. Knight *et al.*, *J. Immunol.* **115**, 595 (1975).
15. P. N. Benfey and N.-H. Chua, *Science* **250**, 959 (1990); W. M. Barnes, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9183 (1990).
16. Leaf blades were cut into segments (2 by 10 mm) and fixed in 3% (w/v) paraformaldehyde, 0.5% (w/v) glutaraldehyde, and 5% (w/v) sucrose in 100 mM sodium phosphate (pH 7.4). After dehydration through a graded ethanol series, leaf segments were infiltrated with xylene, embedded in paraffin, cut into 5-mm sections, and mounted on glass slides for immunochemical staining. The leaf sections were incubated with primary antibodies (affinity-purified rabbit antibody to mouse α chain, which reacts with the A-G hybrid heavy chain, or sheep antibody to rabbit SC) and then with secondary antibodies (goat antibody to rabbit Ig or rabbit antibody to sheep Ig, both labeled with 10-nm gold). The immunogold signal was intensified by silver enhancement.
17. M. B. Hein, Y. Tang, D. A. McLeod, K. D. Janda, A. C. Hiatt, *Biotechnol. Prog.* **7**, 455 (1991).
18. J.-P. Mach, *Nature* **228**, 1278 (1970).
19. L. Winner *et al.*, *Infect. Immun.* **59**, 977 (1991).
20. T. Lehner, J. Caldwell, R. Smith, *ibid.* **50**, 796 (1985); D. Bessen and V. A. Fischetti, *J. Exp. Med.* **167**, 1945 (1988); J. K.-C. Ma, M. Hunjan, R. Smith, C. Kelly, T. Lehner, *Infect. Immun.* **58**, 3407 (1990).
21. M. Kilian, J. Mestecky, M. W. Russell, *Microbiol. Rev.* **52**, 296 (1988).
22. B. J. Underdown and K. J. Dorrington, *J. Immunol.* **112**, 949 (1974); J. Mestecky and J. R. McGhee, *Adv. Immunol.* **40**, 153 (1987).
23. S. G. Rogers, H. J. Klee, R. B. Horsch, R. T. Fraley, *Methods Enzymol.* **153**, 253 (1987).
24. G. Apodaca *et al.*, *J. Biol. Chem.* **268**, 20380 (1993).

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